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ASCORBIC ACID DEFICIENCY INHIBITS INTEGRIN EXPRESSION PRIOR TO ITS EFFECT ON COLLAGEN SYNTHESIS IN FETAL RAT PARIETAL BONE CULTURES. D. Ganta*, G. Pastizzo*, M.B. McCarthy and G. Gronowicz. Dept. of Orthopaedics, University of Connecticut, Farmington, CT.

Ascorbic acid deficiency results in underhydroxylation of collagen and an inhibition of collagen synthesis. We investigated the relationship between collagen synthesis and the β_1 integrin subunit of the collagen receptor. Since the α subunit of the collagen receptor in bone has not been conclusively identified, the β_1 was studied. Treatment of 20 day fetal rat parietal bones with 0, 1, 10 and 100 μg/ml of ascorbic acid for 96 hours had no significant effect on collagen synthesis as determined by [^{3}H]proline incorporation into collagenase digestible protein and noncollagen proteins. No significant differences were found in dry weight, DNA synthesis or DNA content. However, significant effects were demonstrated in calcium and hydroxyproline content.

Ascorbic acid (μg/ml)	Calcium (μg/bone)	Hydroxyproline (μg/bone)
100	17.6±0.8	41.0±2.0
10	16.5±0.8	17.3±3.6*
1	11.3±0.5*	20.5±6.5*
0	12.3±0.7*	9.5 ±3.5*

(*p<0.05 compared to 100 μg/ml)

Light microscopy demonstrated that preosteoblasts and osteoblasts were swollen and no longer contiguous in bones treated with 0, 1 and 10 μg/ml ascorbic acid compared to control bones treated with 100 μg/ml. The osteoid seam was wider in the ascorbic acid-deficient bones. Immunofluorescence staining for the β_1 integrin was found primarily in the osteoblast layer overlying the bone in controls. Staining was markedly diminished in a dose-dependent manner in bones treated with 0, 1 and 10 μg/ml ascorbic acid.

These results demonstrate that Type I collagen synthesis and the collagen integrin are independently regulated in bone culture. In addition, integrins may be able to detect the conformation of collagen fibrils in the matrix and alter receptors on the cell surface accordingly.

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SEQUENCES THAT MEDIATE THE INDUCTION OF OSTEOCALCIN GENE TRANSCRIPTION BY rhBMP-2. K.Goto, Y.Rosen, J.M.Wozney, H.M. Kronenberg and M.B. Demay. Endocrine Unit, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114 and Genetics Institute Inc., Cambridge, MA 02140.

rhBMP-2 induces the differentiation of mesenchymal cells into osteoblastic cells which express the osteoblast-specific protein osteocalcin. Identification of the sequences in the osteocalcin gene responsible for this induction will permit the characterization of factors involved in the developmental regulation of osteocalcin gene expression and in osteoblast differentiation. It has previously been shown that osteocalcin-CAT fusion gene expression can be induced by rhBMP-2 in transient gene expression assays in MLB13MYC clone 17(C17) cells. The DNA sequences from -1750 to +84 are sufficient for this induction. Deletion of the sequences between -522 and -428 results in the abolition of responsiveness to rhBMP-2. To further characterize this region, osteocalcin-CAT fusion genes containing the sequences from -522 to -455, -522 to -341 and -458 to -403 were ligated into an osteocalcin promoter-CAT fusion gene and transfected into C17 cells. After 72 hours of treatment with 250ng/ml of rhBMP-2, CAT activity was induced 2.0-fold in fusion genes containing the sequences from -522 to -341, and -458 to -403. No induction was observed in fusion genes containing the sequences from -522 to -455.

To determine whether rhBMP-2 induced proteins which interacted with these sequences, nuclear extracts were prepared from untreated C17 cells and C17 cells treated with 100ng/ml of rhBMP-2 for 72 hours. When the sequences from -458 to -403 were labeled and incubated with these extracts, unique protein-DNA complexes were observed in the extracts prepared from rhBMP-2 treated cells. The major retarded signal was abolished with 10-fold molar excess of oligonucleotides containing the sequences from -430 to -403, -458 to -418, and -458 to -410, but not with the sequences from -458 to -433 or the 1,25$(OH)_2D_3$ response element (-458 to -442).

These data demonstrate that the sequences from -458 to -403 are sufficient for rhBMP-2 induction. Furthermore, rhBMP-2 induces a protein which binds to the sequences in this oligonucleotide, downstream from the 1,25$(OH)_2D_3$ receptor binding site.

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CHARACTERIZATION OF THE MAJOR NON-COLLAGENOUS PROTEINS IN CHICKEN BONE: IDENTIFICATION OF A NOVEL 60 kDa ACIDIC PROTEIN. **Y. Gotoh, M.J. Glimcher and L.C. Gerstenfeld. Laboratory for the Study of Skeletal Disorders and Rehabilitation, Children's Hospital and Harvard Medical School, Boston, MA 02115**

In order to isolate and characterize the major non-collagenous proteins of chicken bone, a serial extraction procedure was developed by which bone powder was sequentially treated by 4M guanidine HCl (G), followed sequentially by 0.3 M HCl (H), neutral high salt 1M NaCl (NS), and 4M guanidine HCl (NG). This procedure specifically extracted proteins of differing solubility such that osteocalcin (OC) was solubilized in H>NS; osteopontin (OPN) was exclusively extracted in H; α2 HS glycoprotein was extracted in NS>H; bone sialoprotein (BSP) was extracted in NS>H; osteonectin (ON) was extracted in NS>NG, however it was not soluble at all in acidic conditions; and collagen was extracted in NS>NG>H. Amino terminal sequence analysis was performed to identify each of these proteins. Additional internal sequence was obtained for ON demonstrating that the avian form of this protein is over 80% conserved for the available sequence obtained for the 10% of the total sequence presently available. During the course of these studies, a novel ~60 kDa protein was identified showing a unique NH_2 terminal sequence D D P S F D S L G G R H S E G T S. This protein has a high acidic amino acid composition and amino acid analysis indicated that it was similar in composition to other acidic glycoproteins in bone. However, it contained no cysteine and had a lower asp than glu content. By immunological reactivity, it was not recognized by polyclonal antibodies to either BSP or OPN and did not react with a monoclonal antibody to decorin. This protein was not present in chicken serum based on negative reactivity with a polyclonal antibody directed to itself and no comparable sequence was found in the GenBank or EMBL protein sequence database. In summary, these data provide the first sequence for a non-mammalian form of ON and identify a novel acidic bone protein.

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BONE MARROW DEVELOPMENT AND ITS RELATIONSHIP TO BONE FORMATION IN VIVO: A histological study using an implantable titanium device in rabbits. H. Zhou*, [1]P. Choong*, [2]S.T. Chou*, [1]P. Aspenberg*, T.J. Martin, and K.W. Ng. Department of Medicine, The University of Melbourne, St Vincent's Hospital, Fitzroy, Victoria, 3065, Australia. [1]Lund University Hospital, S-221 85 Lund, Sweden. [2]Repatriation General Hospital, Heidelberg, Victoria 3081, Australia.

During embryogenesis, the creation of marrow sinusoids results from the coupled processes of osteogenesis and osteoclastic resorption. Influx of marrow cellular elements follow the formation of bone during endochondral as well as intramembranous ossification.

We set out to further define the relationship between marrow development and bone formation by implanting an intraosseous titanium device into the tibiae of rabbits. A hollow channel is incorporated into the device into which tissue can grow, and the histological sequence of events was observed over 7 weeks.

The channel was in direct continuity with the marrow cavity and isolated from the endosteum. Therefore, immediate marrow regeneration was expected to follow dissolution of the blood clot. Instead at 2W, the first rod-shaped piece of histological tissue consisted of spindle-shaped cells in the centre, flanked at both ends by islands of *osseous tissue* including osteoblasts and osteoclasts. Ingrowth of bone reached the center of the specimen by 4W. However, osteoclastic resorption continued unabated and the quantity of trabecular bone began to diminish so that by 7W, only a thin layer of cortical bone remained. Beginning at 3W, neocapillaries became visible in the intertrabecular spaces. The marrow cavity progressively enlarged with time to be populated by hemopoietic elements.

Although the opportunity existed for immediate marrow regeneration, formation of a marrow cavity and its hemopoietic elements was still preceded by bone formation. Our results provide strong evidence for the primacy of bone formation over marrow development.

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Regulation of Neural Cell Adhesion Molecule and L1 by the Transforming Growth Factor-β Superfamily

SELECTIVE EFFECTS OF THE BONE MORPHOGENETIC PROTEINS*

(Received for publication, August 2, 1993, and in revised form, September 3, 1993)

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The transforming growth factor-β (TGF-β) superfamily plays a role in embryogenesis and regeneration. We have reported that osteogenic protein-1 (OP-1) promotes cell aggregation and induces the expression of the neural cell adhesion molecules N-CAM and L1 in proliferating neuroblastoma × glioma hybrid NG108-15 cells (Perides, G., Safran, R. M., Rueger, D. C., and Charness, M. E. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 10326–10330; Perides, G., Hu, G., Rueger, D. C., and Charness, M. E. (1993) *J. Biol. Chem.* 268, 25197–25205). Here we show that the structurally homologous bone morphogenetic proteins (BMP) BMP-2 and BMP-4 are 10–50-fold more potent in these actions than the subfamily comprising BMP-5, BMP-6, and OP-1 (BMP-7). In contrast, members of the TGF-β subfamily, activin-A, inhibin-A, and 29 additional growth factors and cytokines did not induce N-CAM. The addition of serum to cells growing in serum-free medium caused a concentration-dependent increase in N-CAM and L1 expression; however, serum did not potentiate the induction of N-CAM and L1 by 40 ng/ml OP-1. These findings suggest the presence in NG108-15 cells of a BMP-2/BMP-4 receptor that discriminates subtle differences in structure among homologous members of the TGF-β superfamily. An endogenous ligand for this receptor may be present in serum.

The TGF-β[1] superfamily plays an important role in the development, differentiation, and repair of diverse tissues (1). This superfamily is defined by a common structural element, a 7–9 cysteine domain in the C-terminal region of the mature protein (1). Based on sequence homology in this domain, the TGF-β superfamily has been subdivided into several subfamilies (1–6): the TGF-β subfamily (TGF-β1, -2, -3, -4, -5); the inhibin/activin subfamily (inhibin-A, inhibin-B, activin-A, and activin-B); Müllerian inhibiting substance; and the *dpp*/Vg-related subfamily (decapentaplegic complex (*dpp*) and Vgr/60A from *Drosophila melanogaster*, Vg-1 from *Xenopus laevis*, Vgr-1 and GDF-1 from mouse, osteogenic proteins-1 and 2, the bone morphogenetic proteins (BMPs) 1 through 7, and dorsalin-1). The BMP subfamily can be further subdivided into two subgroups: BMP-2/BMP-4 and BMP-5/BMP-6/BMP-7 (OP-1) (7). Various members of the *dpp*/Vg-1 subfamily are morphogenetically active in the developing nervous system and in bone. For example, the BMPs stimulate endochondral bone formation (8), but have also been identified as morphogens and differentiation factors in neural cells (3, 4, 9, 10).

Cell adhesion molecules are important mediators of cell-cell interactions during embryogenesis and tissue repair (11). Indeed, the neural cell adhesion molecule N-CAM is induced by TGF-β in 3T3 fibroblasts(12), by activin in developing limb buds (13) and by OP-1 (BMP-7) in neuroblastoma × glioma NG108–15 hybrid cells (10). These observations imply that a distinct signaling pathway may couple the TGF-β superfamily to the induction of cell adhesion molecules in mesenchymal and neural tissue. The ligand specificity of the receptor or receptors mediating this response is unknown. Here we demonstrate that the BMPs differentially induce morphological changes and increase the expression of N-CAM and L1 in NG108–15 cells, whereas the TGF-βs, activin-A, inhibin-A, and a large number of other growth factors and cytokines are inactive.

EXPERIMENTAL PROCEDURES

Materials—Rat monoclonal antibody (mAb) H28.123 against N-CAM (14) was purchased from AMAC Inc. (Westbrook, ME) and mAb 5B8 from the Developmental Studies Hybridoma Bank (Iowa City, IA). mAb 74–5H7 against L1 (15) was a generous gift from Dr. V. Lemmon, Case Western Reserve, Cleveland, OH. ^{125}I-Goat anti-rat antibody was obtained from ICN Biomedicals (Irvine, CA), and horseradish peroxidase-conjugated goat anti-mouse was from TAGO (Burlingame, CA). Fetal bovine, newborn calf, and adult bovine sera were purchased from Intergen (Purchase, NY). Epidermal growth factor (EGF) and 2.5 S nerve growth factor (NGF) were purchased from Life Technologies, Inc. Recombinant human OP-1 (hOP-1) was kindly provided by Dr. David C. Rueger, Creative BioMolecules (Hopkinton, MA) and was isolated as described (16). Generous gifts of additional recombinant growth factors were provided as follows: recombinant human BMP-2, BMP-4, BMP-5, and BMP-6 were from Dr. John Wozney, Genetics Institute (Cambridge, MA); recombinant human activin-A and inhibin-A were from Dr. Ralph Schwall, Genentech (South San Francisco, CA); *dpp* and 60A were from Dr. Michael Hoffman, University of Wisconsin (Madison, WI); and the remaining human recombinant growth factors and cytokines were from Dr. Monica Tsang, R & D Systems (Minneapolis, MN). All other chemicals were purchased from Sigma and were of reagent grade.

Cell Culture—NG108–15 cells of passages 21–30 were cultured in serum-free medium as described (10). Prior to subculture, the cells were mechanically dispersed until single. Cells were photographed at a mag-

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[1] The abbreviations used are: TGF-β, transforming growth factor-β; BMP, bone morphogenetic proteins; *dpp*, decapentaplegic complex; EGF, epidermal growth factor; FGF, fibroblast growth factor; hOP-1, recombinant human osteogenic protein-1; IgCAM, immunoglobulin superfamily cell adhesion molecule; mAb, monoclonal antibody; N-CAM, neural cell adhesion molecule; NGF, nerve growth factor; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

nification of × 200 under phase contrast microscopy. Because BMP-treated cells grew in clusters, some × 200 fields contained clusters of large numbers of cells while others showed few; therefore, the photographs do not accurately reflect cell number, which was not influenced by BMP treatment.

Morphogenetic Actions of hOP-1—Two days after the addition of recombinant growth factors, subconfluent (<50%) cells viewed at × 100 magnification were scored for the presence of cell clusters. A cell cluster was defined as a group of three or more cells that adhered to each other along at least one-quarter of the cell diameter. Adherent pairs of cells were excluded from this definition because many consisted of post-mitotic pairs which, in the absence of growth factors, eventually separated. The percentage of cells in clusters was calculated by dividing the number of cells present in clusters by the total number of cells (150–200) in each of two randomly selected fields. Values obtained for the two fields were averaged and differed by up to 15%.

ELISA for N-CAM—Levels of N-CAM were quantitated using an ELISA as described (17, 18), with the following modifications. To determine the linearity of the assay with respect to cell number, NG108–15 cells were cultured for 3 days in 75-cm² flasks in the absence and presence of 40 ng/ml hOP-1. 18 h before assay, duplicate samples of 6,000–90,000 cells/well were plated in poly-D-lysine-coated 96-well trays in the continued presence of hOP-1. To determine the effects of growth factors on N-CAM expression, duplicate samples of cells were plated on 96-well trays at a density of 4,500 cells/well and incubated for 24–48 h in serum-free medium. Following the addition of growth factors, the medium was replaced daily. N-CAM was assayed after 3 days of treatment with growth factors. Cells were fixed by incubating twice for 30 min at 4 °C in 100 µl of ice-cold methanol, washed three times with PBS, blocked with 10% non-fat dry milk in PBS for 1 h, and washed three additional times with PBS. Cells were then incubated for 1 h at room temperature in 100 µl of PBS containing 5% fetal bovine serum supplemented with the primary antibody (1:2,000 dilution of anti-N-CAM mAb 5B8), washed three times in PBS, and incubated an additional 1 h at room temperature in 100 µl of PBS containing 5% fetal bovine serum supplemented with the secondary antibody (1:1,000 dilution of goat anti-mouse IgG and IgM conjugated to horseradish peroxidase). The cells were washed an additional three times with PBS. Color was developed by adding 100 µl of 80 mg/dl of 5-aminosalicylic acid in 0.02 M monobasic sodium phosphate, pH 6.0, containing 0.02% H_2O_2, and the optical density was measured at 490 nm. The reaction was linear with respect to cell number between 6,000 and 50,000 cells/well. Nonspecific color reaction, determined by omitting the primary antibody in duplicate wells, was subtracted from the total optical density for each sample. All values are expressed relative to control values measured on the same tray.

Western Blot Analysis—Western blots of total cell protein were performed using mAb H28.123 for N-CAM and mAb 74–5H7 for L1, as described (10, 19).

RESULTS AND DISCUSSION

Morphogenetic Activity of the BMPs in NG108–15 Cells—We showed previously that hOP-1 induces the expression of the immunoglobulin superfamily cell adhesion molecules (IgCAM) N-CAM and L1 in dividing NG108–15 cells, leading to a concentration-dependent increase in the percentage of cells present in adherent clusters and epithelioid sheets (10, 19). To determine the morphogenetic activity of other members of the TGF-β superfamily, NG108–15 cells were incubated for 2 days in serum-free medium supplemented with 1 or 10 ng/ml of various BMPs or TGF-β1 and scored for the presence of adherent clusters of cells. At 10 ng/ml, all of the BMPs induced cluster formation (Fig. 1). However, at 1 ng/ml, BMP-2 and BMP-4 were more effective in promoting cell clustering than BMP-5, BMP-6, and OP-1. In contrast, TGF-β1 was morphogenetically inactive at concentrations ranging from 0.01 to 40 ng/ml. OP-1 did not change the rate of NG108–15 cell division (19) and treatment with the BMPs did not significantly affect the protein content per well (BMP-2, 104 ± 13% control; BMP-4, 94 ± 13%; BMP-5, 91 ± 11%; BMP-6, 126 ± 14%; OP-1 (BMP-7) 96 ± 2%; n = 3–5). These data suggest that in NG108–15 cells, the morphogenetic activity of the TGF-β superfamily increases as a function of structural homology with the BMP-2/BMP-4 subfamily and is not related to effects on cell growth. These

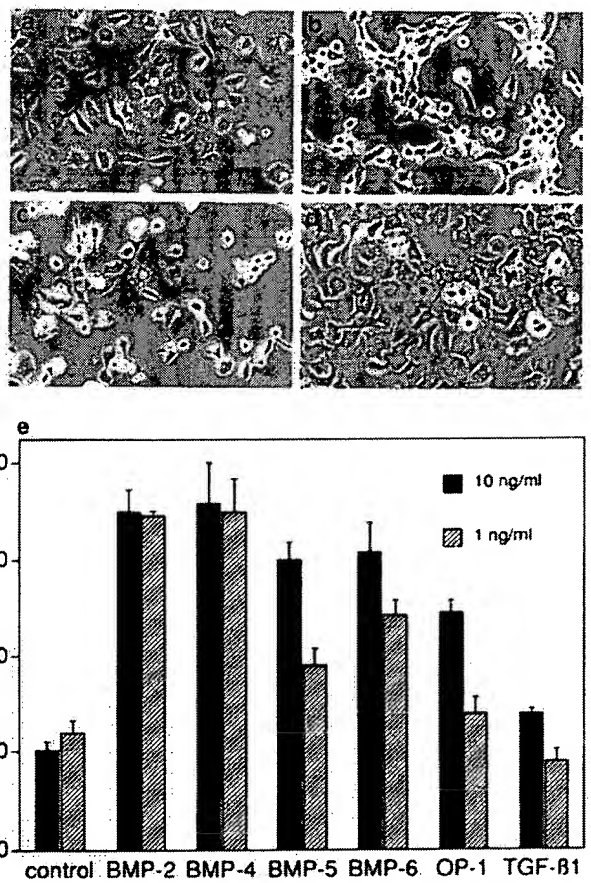

FIG. 1. **Morphological effects of the BMPs in NG108-15 cells.** Photomicrographs of NG108–15 cells cultured for 2 days in serum-free medium in the absence (*a*) and presence of 1 ng/ml of BMP-4 (*b*), 1 ng/ml BMP-6 (*c*), and 40 ng/ml TGF-β1 (*d*). Lower concentrations of TGF-β1 were also morphogenetically inactive. Note that the TGF-β-treated cells, while present at a greater density in the field shown, do not form more clusters than the control cells. *e*, NG108–15 cells were cultured in serum-free medium in the absence and presence of 1 or 10 ng/ml of the indicated recombinant human growth factors. The percentage of cells present in adherent groups of three or more cells (clusters) was determined from two subconfluent fields of at least 150 cells (see "Experimental Procedures"). The total number of cells in the two fields did not differ among control and BMP-treated cells. Shown are the mean ± S.E. percentage of cells present in clusters from three to four independent experiments. The two control values are from duplicate sets of experiments.

experiments also suggest that the morphoregulatory activity of the BMPs is mediated by a distinct receptor(s) from that which recognizes the TGF-β subfamily. Additional evidence for the existence of distinct OP-1 and TGF-β receptors derives from studies in cultured osteoblasts. Whereas both hOP-1 and TGF-β1 promote the proliferation of osteoblasts in culture, only hOP-1 stimulates markers of the osteoblast phenotype (16).

Effects of the TGF-β Superfamily on N-CAM and L1 Expression—The morphoregulatory actions of hOP-1 are mediated in part by the induction of N-CAM and L1 (10, 19). To determine whether the variable morphogenetic activity of the TGF-β superfamily is due to differential induction of IgCAMs, we cultured NG108–15 cells for 3 days in the absence and presence of members of the TGF-β superfamily and measured levels of N-CAM and L1 by ELISA and Western blot analysis. Of several antibodies tested, the best results for the ELISA detection of N-CAM were obtained using mAb 5B8, which recognizes the cytoplasmic domain present in N-CAM-140 and N-CAM-180, but not N-CAM-120. To confirm that the ELISA was linear with

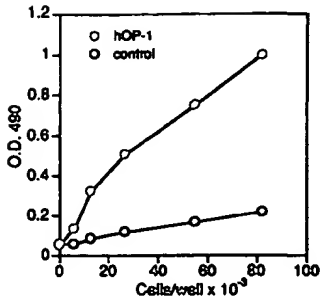

FIG. 2. **ELISA determination of N-CAM levels in control and hOP-1-treated cells.** NG108–15 cells were cultured for 3 days in T75 flasks in the absence and presence of 40 ng/ml hOP-1. 18 h before assay, cells were subcultured at the indicated densities in poly-D-lysine-coated 96-well plastic trays. Cells were fixed and N-CAM levels were determined by ELISA using mAb 5B8. Shown are the mean optical densities from duplicate samples of cells from a representative experiment that was repeated five times with similar results.

respect to cell number, we cultured NG108–15 cells at a density of 6,000–90,000 cells/well in 96-well microtiter plates and measured levels of N-CAM. Fig. 2 indicates that the ELISA was linear within a 15-fold range of cell concentrations. This finding is of interest, because N-CAM levels increase when N2A neuroblastoma cells are plated at a high density, and conditioned medium from N2A neuroblastoma cells increases N-CAM expression in N2A neuroblastoma and 3T3 fibroblast cells (12). The observation that N-CAM levels are linear with respect to NG108–15 cell numbers suggests that in contrast to N2A neuroblastoma cells, NG108–15 cells do not produce an autocrine regulator of N-CAM expression.

The ELISA was also effective in demonstrating a large induction of N-CAM by hOP-1 (Fig. 2), allowing us to use this rapid technique for quantitatively screening the effects of the TGF-β superfamily and other growth factors on the levels of N-CAM. None of the TGF-βs showed significant N-CAM-inducing activity (Fig. 3*a*); in contrast, the BMPs exhibited striking and differential concentration-dependent induction of N-CAM (Fig. 3*b*). Because mAb 5B8 detects only two of the three major N-CAM isoforms induced by hOP-1, the ELISA tended to underestimate the magnitude of N-CAM induction demonstrated by Western blot analysis (10). The potencies of the BMPs in inducing N-CAM correlated with their structural homology. BMP-4 showed half-maximal effectiveness at a concentration of approximately 0.2 ng/ml. The other member of this subfamily, BMP-2, was the next most potent BMP (EC_{50} ~1 ng/ml). In contrast, BMP-5, BMP-6, and OP-1 did not significantly induce N-CAM at concentrations lower than 1 ng/ml and exhibited equivalent effects at higher concentrations (EC_{50} ~10 ng/ml). Western blot analysis confirmed these results and demonstrated a comparable ordering of the BMPs in the induction of L1 (Fig. 3*c*). The fact that the potency of each BMP in inducing IgCAMs was similar to that for promoting cell clustering provides additional evidence that the morphoregulatory actions of the BMPs in NG108–15 cells are mediated by the induction of N-CAM and L1. Activin-A, inhibin-A, NGF, and EGF did not induce any of the major N-CAM isoforms or L1 (Fig. 3*c*).

BMP-2 and BMP-4 are 92% identical in the TGF-β domain; similarly, BMP-5, BMP-6, and OP-1 (BMP-7) show 87–88% amino acid sequence identity (7). These two BMP subgroups differ only slightly from each other (57–61% identity), yet show a 10–50-fold difference in their potency for inducing N-CAM. Our results indicate that a receptor expressed in NG108–15 cells can discriminate a small difference in structure between the two BMP subgroups and fails to recognize activin-A (41% sequence identity with BMP-4), the TGF-βs (32–35% identity BMP-4), and inhibin-A (22% identity BMP-4). Moreover, the same receptor appears to be responsible for the induction of both N-CAM and L1. A careful analysis of IgCAM-inducing activity and sequence homology among additional members of the TGF-β superfamily may allow a precise identification of the molecular domains responsible for the activation of this receptor. Interestingly, *dpp* and 60A, the *Drosophila* homologs of the BMP-2/BMP-4 (75% identity) and BMP-5/BMP-6/OP-1 (69–74% identity) subfamilies, respectively (7, 20), did not induce N-CAM expression in NG108-15 cells (not shown). This implies that the region of the protein that differs only slightly between BMP-4 and *dpp* is particularly important for activating signaling along this pathway.

Effect of Serum and Diverse Growth Factors on Cell Morphology and N-CAM Expression—Serum may contain factors that regulate IgCAM expression: growth in serum increases the expression of N-CAM and decreases the expression of L1 in N2A neuroblastoma cells (12). Small increases in N-CAM expression are also observed when serum-free myotube cultures are incubated with serum or a variety of growth factors (21). To evaluate whether serum regulates the expression of N-CAM in NG108-15 cells, we cultured cells for 3 days in 96-well trays containing serum-free medium supplemented with 0.1–10% fetal bovine, newborn calf, or adult bovine sera. Fig. 4 demonstrates that these sera caused a concentration-dependent increase in the expression of N-CAM that was inversely proportional to the age of the donor. This developmental loss of N-CAM-inducing activity in serum is consistent with previous observations that OP-1 and BMP gene expression declines during development (22, 23). The induction of N-CAM and L1 by serum led to the formation of cell clusters and multilayered cell aggregates; however, in contrast to hOP-1 (19), serum did not cause a small percentage of cells to extend neurites (data not shown). The presence in serum of thrombin and related substances that inhibit neuritogenesis (24) may have opposed the neurite promoting actions of N-CAM.

The strong IgCAM-inducing activity of the BMPs raises the question as to whether these proteins account for the IgCAM-inducing activity of serum. We addressed this question in two ways. First, using the ELISA, we screened a large and diverse number of recombinant morphogens and cytokines for N-CAM inducing activity. Table I shows that among 40 growth factors, only the BMPs exhibited significant N-CAM-inducing activity. The list of inactive factors is significant for the presence of several peptides that promote the survival, differentiation, and morphogenesis of the brain and skeleton, including NGF, EGF, platelet-derived growth factor, fibroblast growth factor-4, interleukin-6, and leukemia inhibitory factor (25, 26). Second, we asked whether the actions of serum and the BMPs are synergistic or additive. NG108–15 cells were cultured for 3 days in serum-free medium supplemented with 0.1–10% fetal bovine serum in the absence and presence of 40 ng/ml hOP-1. Fetal bovine serum produced a concentration-dependent increase in the levels of all three major isoforms of N-CAM and L1 (Fig. 4*b*); however, the induction of IgCAMs by hOP-1 was not potentiated by serum. In contrast, the N-CAM-inducing effects of serum and TGF-β are additive in NIH 3T3 cells (12). Although these experiments do not prove that BMPs are present in serum, they suggest that BMPs or factors that act similarly account for part of the IgCAM-inducing activity of serum.

Morphoregulatory and IgCAM-inducing Activity of the TGF-β Superfamily in Mesenchymal and Neural Tissue—The morphoregulatory actions of the BMPs have been well characterized in bone (8); however, an increasing body of data suggests that these proteins play an important role in the development of the nervous system. Several of the BMPs exhibit unique spatial and temporal patterns of expression in the de-

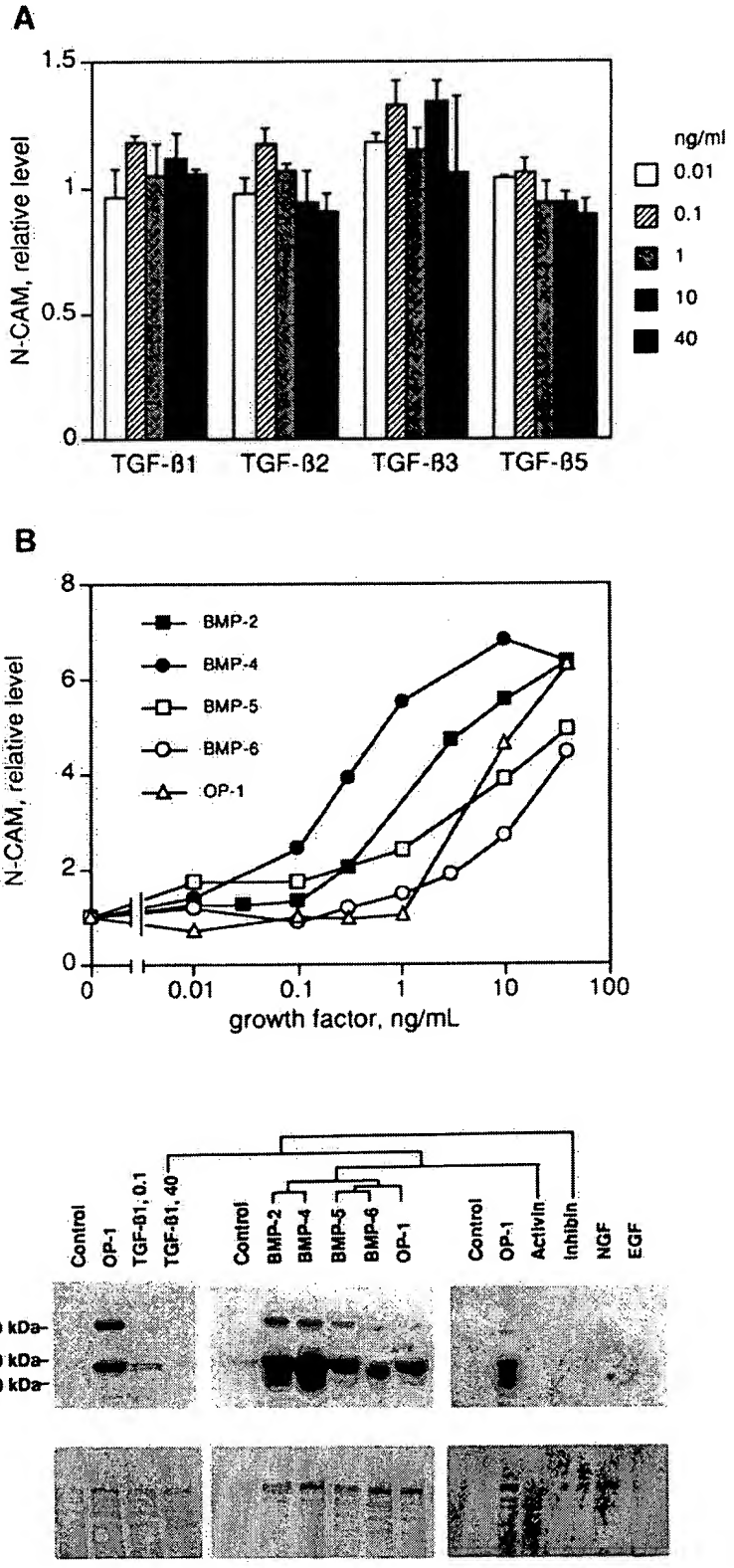

FIG. 3. **Effect of the TGF-β superfamily on N-CAM and L1 expression.** For ELISA determination of N-CAM, NG108–15 cells were plated at 4,500 cells/well in 96-well poly-D-lysine-coated plastic trays and incubated for 3 days in the absence and presence of the indicated concentrations of TGFβs (*A*) and BMPs (*B*). Optical densities for control values determined on the same tray were normalized to 1 and values for the morphogen-treated cells were expressed relative to control values (relative change). Shown are the mean ± S.E. (*A*) or the mean (*B*) values from three to four independent experiments. *C*, NG108-15 cells were cultured in six-well trays for 3 days in the absence (control) and presence of 10 ng/ml of BMP-2, BMP-4, BMP-5, BMP-6, and hOP-1 and 40 ng/ml of activin-A, TGF-β1, inhibin-A, nerve growth factor (NGF), and epidermal growth factor (EGF). Cells were harvested, solubilized in SDS sample buffer, and the proteins were subjected to immunoblot analysis for N-CAM (H28.123 mAb) and L1 (74-5H7 mAb). Shown are representative immunoblots from an experiment that was repeated three times with similar results. The structural homology of the TGF-β superfamily is indicated by a map of amino acid sequence identity in the C-terminal TGF-β domain, as reported by others (7, 20).

veloping nervous system, and some are known to be species homologues of developmental genes in *Drosophila* and *Xenopus*. (27, 28). BMP-4 mRNA is expressed transiently in the floorplate of the diencephalon adjacent to Rathke's pouch, where it is speculated to play an inductive role during pituitary development (29). BMP-4 also stimulates differentiation in PC12 cells (4). BMP-6 mRNA is expressed selectively in the roofplate adjacent to the forebrain and in cells adjacent to the

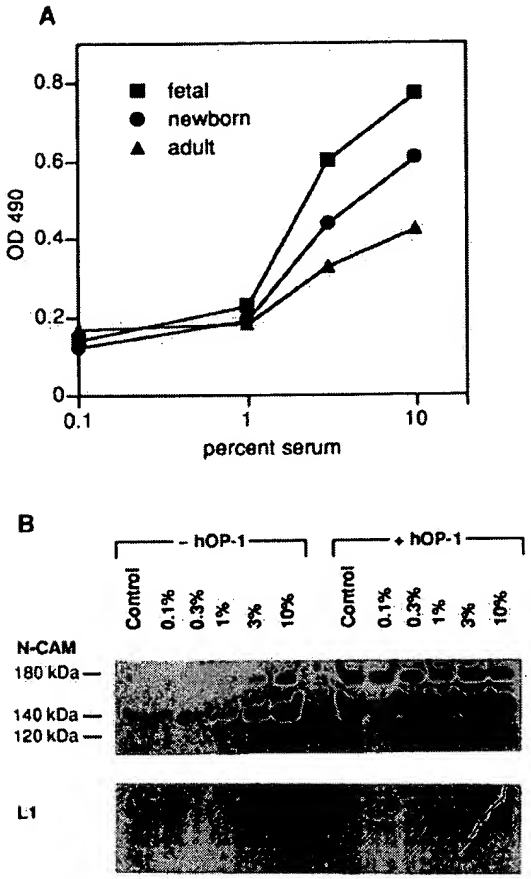

FIG. 4. **Effect of serum on N-CAM and L1 expression in NG108-15 cells.** *a*, NG108-15 cells were cultured in 96-well trays containing serum-free medium in the absence and presence of the indicated concentrations of fetal bovine serum, calf serum, and adult bovine serum. After 3 days cells were subjected to ELISA for N-CAM determination. Shown are the optical densities from a single experiment that was repeated three times with similar results. *b*, NG108-15 cells were cultured for 3 days in 6-well trays in serum-free medium supplemented with 0 (control) to 10% fetal bovine serum in the absence and presence of 40 ng/ml hOP-1. Cells were harvested and subjected to immunoblot analysis for N-CAM and L1. Shown is a representative autoradiograph from an experiment that was repeated three times with similar results.

floor plate along the anterior-posterior neuraxis (6, 29). BMP-4, BMP-5, and BMP-6 mRNA are not detected in the brains of 2-week post-natal and adult mice; however, OP-1 (BMP-7) mRNA is present in both (2), suggesting that OP-1 may also play a role in the adult nervous system.

Other members of the TGF-β superfamily are also active in mesenchymal and neural cells. Activin induces N-CAM and stimulates chondrogenesis in developing limb buds (13), but also induces somatostatin immunoreactivity in ciliary ganglion neurons (30) and is a neuronal survival factor (31). Dorsalin-1 is a novel member of the *dpp*/Vg-related subfamily that shows 55% sequence identity with BMP-4 and is selectively expressed in dorsal regions of the neural tube (3). Dorsalin-1 promotes the differentiation and migration of neural crest cells and inhibits the differentiation of motor neurons (3). Dorsalin-1 also induces alkaline phosphatase activity in W-20-17 osteoblast cells (3), demonstrating that like OP-1, BMP-4, and activin, dorsalin-1 has biological activity in bone and in brain.

It is unknown how most members of the TGF-β superfamily produce their morphogenetic effects. We hypothesize that some of these morphogens act in mesenchymal and neural tissue by inducing the expression of IgCAMs at critical periods in development and tissue repair. OP-1 stimulates endochondral bone formation (16), and N-CAM is transiently expressed in osteoblasts during intramembranous and endochondral bone formation (32). It is not yet known whether the induction of IgCAMs mediates the osteogenic actions of the BMPs or the neural actions of dorsalin and the BMPs. However, it is possible that a conserved signaling pathway couples some members of the TGF-β superfamily to the induction of IgCAMs in mesenchymal and neural cells, with different, tissue-specific effects.

TABLE I

Effect of growth factors and cytokines on N-CAM expression

NG108-15 cells were cultured in 96-well trays containing serum-free medium in the absence and presence of 40 ng/ml of the indicated growth factors and cytokines. After 3 days cells were subjected to ELISA for N-CAM determination. Optical densities for control values determined on the same tray were normalized to 1, and values for the morphogen-treated cells were expressed relative to control values (relative OD). Shown are the mean ± S.E. relative OD values for the indicated number of experiments (parentheses). Except for the BMPs, similar results were obtained when the factors were tested at their half-maximal biological activity (ED_{50}) and one tenth of ED_{50} (as listed in the manufacturers specification sheets). All growth factors and cytokines used were recombinant human except TGF-β2 (porcine), TGF-β3 (chicken), and TGF-β5 (*Xenopus*).

Growth factor (40 ng/ml)	Relative OD, mean ± S.E. (*n*)
BMP-2	6.01 ± 0.66 (5)
BMP-4	6.38 ± 1.85 (5)
BMP-5	4.93 ± 2.03 (2)
BMP-6	4.43 ± 0.54 (5)
OP-1 (BMP-7)	6.30 ± 1.69 (4)
Activin-A	0.99 ± 0.08 (5)
Transforming growth factor-β1	1.05 ± 0.02 (3)
Transforming growth factor-β2	0.91 ± 0.07 (4)
Transforming growth factor-β3	1.06 ± 0.30 (3)
Transforming growth factor-β5	0.89 ± 0.07 (3)
Inhibin-A	1.01 ± 0.06 (5)
Nerve growth factor (2.5 S)	1.02 ± 0.06 (6)
Fibroblast growth factor-4	0.98 ± 0.38 (2)
Epidermal growth factor	0.95 ± 0.10 (4)
Platelet-derived growth factor-AA	1.09 ± 0.04 (2)
Platelet-derived growth factor-BB	1.09 ± 0.12 (2)
Platelet-derived growth factor-AB	1.08 ± 0.25 (2)
Interleukin-1α (IL-1α)	0.89 ± 0.09 (2)
Interleukin-1β	0.81 ± 0.00 (1)
Interleukin-2	1.02 ± 0.02 (2)
Interleukin-3	1.00 ± 0.23 (2)
Interleukin-4	1.09 ± 0.06 (2)
Interleukin-5	0.93 ± 0.13 (2)
Interleukin-6	0.80 ± 0.10 (2)
Interleukin-7	0.80 ± 0.02 (2)
Interleukin-8	0.83 ± 0.11 (2)
Interleukin-9	0.91 ± 0.10 (2)
Interleukin-10	0.91 ± 0.07 (2)
Interleukin-11	0.80 ± 0.03 (2)
Interleukin-1 ra	1.04 ± 0.09 (2)
Interleukin-γ	1.00 ± 0.07 (2)
GROα/melanoma growth-stimulating activity	1.06 ± 0.15 (2)
Leukemia-inhibitory factor	1.19 ± 0.05 (2)
Oncostatin M	0.78 ± 0.20 (2)
Tumor necrosis factor-α	0.90 ± 0.12 (2)
Tumor necrosis factor-β	0.83 ± 0.06 (2)
Macrophage inflammatory protein-1α	0.89 ± 0.13 (2)
Macrophage inflammatory protein-1β	0.92 ± 0.17 (2)
Monocyte chemotactic protein-1	0.85 ± 0.30 (2)
RANTES (regulated upon activation, normal T-cell expressed and presumably secreted)	0.87 ± 0.07 (2)

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Human Osteogenic Protein-1 Induces both Chondroblastic and Osteoblastic Differentiation of Osteoprogenitor Cells Derived from Newborn Rat Calvaria

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Abstract. Osteogenetic protein-1 (OP-1), a member of the TGF-β superfamily, induces endochondral bone formation at subcutaneous sites in vivo and stimulates osteoblastic phenotypic expression in vitro. Primary cultures of newborn rat calvarial cells contain a spectrum of osteogenic phenotypes ranging from undifferentiated mesenchymal osteoprogenitor cells to parathyroid hormone (PTH)-responsive osteoblasts. We examined whether treatment of this cell population with recombinant human osteogenic protein-1 could induce chondrogenesis in vitro. Markers of chondroblastic versus osteoblastic differentiation included alcian blue staining at pH 1, alkaline phosphatase-specific activity, osteocalcin radioimmunoassay, and expression of collagen mRNAs. 6 d of treatment (culture days 1-7) with 4-100 ng OP-1/ml caused dose-dependent increases in alcian blue staining intensity and alkaline phosphatase activity (4.7- and 3.4-fold, respectively, at 40 ng/ml), while osteocalcin production decreased twofold. Clusters of round, refractile, alcian blue-stained cells appeared by day 3, increased in number until day 7, and then became hypertrophic and gradually became less distinct. Histochemically, the day 7 clusters were associated with high alkaline phosphatase activity and became mineralized. mRNA transcripts for collagen types II and IX were increased by OP-1, peaking at day 4, while type X collagen mRNA was detectable only on day 7 in OP-1-treated cultures. Delay of OP-1 exposure until confluence (day 7) amplifies expression of the normal osteoblastic phenotype and accelerates its developmental maturation. In contrast, early OP-1 treatment commencing on day 1 strongly amplifies chondroblastic differentiation. In the same protocol, TGF-β1 alone at 0.01–40 ng/ml fails to induce any hypertrophic chondrocytes, and in combination with OP-1, TGF-β1 blocks OP-1–dependent chondroinduction. OP-1 is believed to act on a subpopulation of primitive osteoprogenitor cells to induce endochondral ossification, but does not appear to reverse committed osteoblasts to the chondrocyte phenotype.

Bone matrix contains various growth factors which control bone formation and resorption, and these factors clearly play important roles in the development and growth of cartilage and bone (Hauschka et al., 1986; Centrella et al., 1987; Hauschka, 1990). Implantation of demineralized bone matrix at intramuscular or subcutaneous sites induces cartilage and bone formation (Reddi and Huggins, 1972; Urist, 1965). Recently, several proteins involved in ectopic bone formation have been purified from bovine demineralized bone matrix, allowing the cloning of their full-length cDNAs and discovery of related cDNAs: bone morphogenetic proteins (BMPs)[1] (BMP-1 through BMP-7) (Wong et al., 1988; Wozney et al., 1988; Celeste et al., 1990); osteogenin (BMP-3) (Luyten et al., 1989); and osteogenic protein-1 (OP-1/BMP-7) (Özkaynak et al., 1990; Sampath et al., 1990) and OP-2 (Özkaynak et al., 1992). cDNA sequencing has revealed that these proteins are members of the superfamily of TGF-β–related proteins which share a distinctive pattern of seven cysteine residues in their COOH-terminal domains (Celeste et al., 1990; Ozkaynak et al., 1990; Wozney et al., 1988).

The members of the TGF-β superfamily play diverse and significant roles in growth and differentiation. For example, *Drosophila* decapentaplegic complex (dpp) is involved in embryonic dorsal/ventral polarity (Segal and Gelbart,

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1. *Abbreviations used in this paper*: BMP, bone morphogenetic protein; OP, osteogenic protein; TFA, trifluoracetic acid.

1985). Vg-1 is thought to act as an intercellular signal for mesoderm induction in *Xenopus* (Rebagliati et al., 1985). Vgr-1 (BMP-6) and BMP-2 (BMP-2a) expression has been analyzed in various tissues during mouse embryogenesis (Lyons et al., 1989). BMPs and OP-1 are thought to be involved in cartilage and bone development.

Formation of new bone is induced when OP-1 and other BMPs are implanted with carriers in nonbony sites in rats. This induction always involves the cascade of de novo endochondral bone formation: cartilage is induced and becomes calcified before being replaced by mineralized bone (Reddi, 1981; Sampath et al., 1992). Several in vitro studies have addressed the mechanism of action of bone-inducing proteins. These studies have shown that BMPs and OP-1 have two osteogenic effects. First, they enhance the osteoblastic characteristics of osteoblast-like cells, including MC3T3-E1 mouse calvaria-derived osteoblasts (BMP-2 and BMP-3) (Hiraki et al., 1991; Takuwa et al., 1991; Vukicevic et al., 1990), and rat calvaria-derived primary osteoblast-like cells (BMP-3 [Vukicevic et al., 1989]; BMP-4 [Chen, T., et al., 1991]; OP-1 [Sampath et al., 1992]). Second, these factors induce osteoblast phenotypic expression by osteoprogenitor cells such as rat calvaria-derived cells (BMP-2; Yamaguchi et al., 1991), bone marrow stromal cells (BMP-2; Thies et al., 1991), and C3H10T1/2 mouse embryo-derived mesenchymal cells (BMP-2; Katagiri et al., 1990). Other studies focussing on cartilage showed that native preparations of BMP-2/BMP-3 (Hiraki et al., 1991), BMP-3 alone (Vukicevic et al., 1989), and BMP-4 (Luyten et al., 1992) could enhance cartilage characteristics of cultured articular chondrocytes. Osteogenin (BMP-3) and BMP-4 have also been noted to induce the cartilage phenotype in chondrocyte precursor chick limb bud cells (Carrington et al., 1991; Chen, P., et al., 1991), and to promote reexpression of cartilage phenotype by dedifferentiated articular chondrocytes (Harrison et al., 1991). However, there has been no evidence until our study that OP-1 or other BMPs could induce chondrogenesis in noncartilage cells in vitro.

In contrast to the endochondral development of long bones, membranous flat bones of the skull (e.g., calvaria) develop by intramembranous ossification, bypassing the calcified cartilage stage characteristic of the endochondral process. In postfetal life, however, when injury or insult occurs, skull bones repair through a cellular process which includes both intramembranous and endochondral ossification (Marden et al., 1993). Might OP-1 and other BMPs play an intrinsic role in osseous healing? It is possible that chondrocytes and osteoblasts are developed by differentiating from the same common lineage of uncommitted mesenchymal osteoprogenitor cells, although the identity of the OP-1 target cell and the precise lineage leading to chondrocyte and osteoblast phenotypes is not clearly established.

While exploring the temporal dependence of OP-1 regulation of primary rat osteoblasts in vitro, we observed strong chondroinduction resulting from early OP-1 treatment, whereas if left untreated, these cultures would have naturally progressed to mature, mineralizing osteoblasts without evidence of cartilage. We hypothesized that OP-1 has chondroinductive effects on a subset of uncommitted osteoprogenitor cells derived from neonatal rat calvaria, and that the combined OP-1 effects of chondroinduction (this study) and the known osteogenic stimulation of committed osteoblasts (Sampath et al., 1992) could account for the in vivo observations that OP-1 induces the cascade of endochondral ossification.

Materials and Methods

Preparation of Recombinant Human OP-1

Recombinant human OP-1 (OP-1) was prepared as described previously (Sampath et al., 1992). Briefly, the full-length OP-1 cDNA was inserted into a mammalian expression vector and transfected into dhfr(−) CHO cells which were subjected to methotrexate-mediated gene amplification. The resulting selected cell line was cultured in roller bottles, and the conditioned media was collected. OP-1 was purified from conditioned media using three chromatography steps: S-Sepharose, phenyl-Sepharose, and reverse phase HPLC (Sampath et al., 1992). Mature OP-1 is a glycosylated 36-kD homodimer of 139-amino acid residue chains. Stock solutions of OP-1 were prepared in 50% ethanol, 0.1% trifluoroacetic acid (TFA) and standardized for protein concentration by amino acid analysis.

Cell Culture

Calvaria from 1-d-old Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were dissected, and five sequential 20-min digests (I-V) were performed on suture-free calvarial fragments at 37°C in Hefley's buffer (Hefley et al., 1981) containing 2 mg/ml collagenase B (Boehringer-Mannheim Corp., Indianapolis, IN) and 0.25% trypsin (GIBCO BRL, Gaithersburg, MD). Single cell suspensions obtained from digests III to V were pooled. In some experiments, calvaria were separated into four anatomical regions (frontal bone, parietal bone, occipital bone, and sutures) and digested separately as described above. Cells were plated on day 0 in MEM (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FBS (Bioproducts for Science Inc., Indianapolis, IN) and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin; GIBCO BRL) in 12-multiwell plates (Elkay, Shrewsbury, MA) at a density of 1.2×10^5 cells per 1-ml well. Treatment with OP-1 and 50 μg/ml L-ascorbic acid (Sigma Chemical Co.) was started 1 d after plating, and medium was replaced completely every 3 d beginning on day 1. To examine the effect of OP-1 on mineralization, 10 mM β-glycerophosphate (Sigma Chemical Co.) was added to the medium from day 1.

All OP-1 additions to cultured cells involved rapid dispersal of $\leq 10^{-3}$ vol of OP-1 stock solution (in 50% ethanol, 0.1% TFA) into the culture medium immediately before refeeding cells; solvent vehicle alone was added to control medium.

Human, recombinant TGF-β1 (R & D Systems, Minneapolis, MN) was prepared at 20 μg/ml in 4 mM HCl containing 1 mg/ml BSA. TGF-β1 or control vehicle was added to medium before refeeding.

For experiments examining the effect of OP-1 on chondrogenesis versus osteogenesis, primary cultures were compared with subcultivated cell cultures. Subcultivation was performed by trypsin digestion of day 7 confluent primary cultures grown in the absence of OP-1 and ascorbic acid. The washed cell suspension was diluted to 1.2×10^5 cells/ml and replated under the same conditions as the primary culture.

Cell Growth

Effects of OP-1 on cell growth were examined by determining the rate of [^{3}H]thymidine incorporation into total acid-insoluble DNA and by measurement of total DNA in cell layers.

DNA synthesis rates were determined after every 3 d of treatment with OP-1 by adding [^{3}H-methyl]thymidine (50 Ci/mmol; Amersham Corp., Arlington Heights, IL). Cells were labeled with 2 μCi/ml of [^{3}H]thymidine for 4 h before termination of the culture. The 5% TCA-precipitated radioactive DNA in the cell layer was solubilized with 0.5 N NaOH and quantitated by liquid scintillation counting.

DNA content was determined by fluorometric assay (Vytasek, 1982). Cells were precipitated with 5% TCA, dissolved in 1 N NaOH, hydrolyzed in 1 N perchloric acid at 70°C for 20 min, and incubated with diaminobenzoic acid dihydrochloride at 37°C for 1 h. DNA concentration was determined against a calf thymus DNA standard (Sigma Chemical Co.) by measuring the fluorescence emission at 500 nm during excitation at 408 nm using a Perkin Elmer LS-5 fluorescence spectrophotometer (Perkin-Elmer Corp., Norwalk, CT).

Measurement of Chondrogenesis

The degree of chondrogenesis was evaluated by staining with alcian blue. Cultures were washed with PBS, fixed 10 min with 4% paraformaldehyde, stained with 0.5% alcian blue (Fluka, Ronkonkoma, NY) in 0.1 N HCl, pH 1.0, overnight, and rinsed with distilled water. The quantity of sulfated glycosaminoglycan representative of cartilage matrix was estimated by measuring the amount of extractable dye (San Antonio and Tuan, 1985). Alcian blue-stained cultures were extracted with 6 M guanidine-HCl for 2 h at room temperature. Optical density of the extracted dye was measured at 650 nm in a 96-well plate reader (Molecular Devices, Menlo Park, CA).

Alkaline Phosphatase Activity

Cell layers were extracted with 20 mM TBS, pH 7.4, containing 1% Triton X-100 and stored at −20°C until the assay. Enzyme activity was assayed with 10 mM (final) *p*-nitrophenyl phosphate as a substrate in 50 mM sodium carbonate buffer, pH 10, containing 5 mM $MgCl_2$. After 10 min of incubation at 37°C, the reaction was stopped with 0.5 N NaOH and absorbance was measured at 405 nm. Protein in each sample was determined with the BCA protein assay kit (Pierce, Rockford, IL). Alkaline phosphatase specific activity is presented as μmol *p*-nitrophenol produced/min/μg protein.

Osteocalcin Radioimmunoassay

Rat osteocalcin levels in the media were measured by radioimmunoassay as described previously (Gundberg et al., 1984). Conditioned medium samples were collected and stored at −20°C. Radioimmunoassay was performed using goat anti-rat osteocalcin serum as first antibody and donkey anti-goat IgG as second antibody. The intraassay and interassay variance was ± 5% and ± 8%, respectively. Data are expressed as total ng rat osteocalcin in the 3-d-conditioned medium per μg cell layer DNA.

Histochemical Analysis

Cell layers were rinsed with PBS or 0.9% NaCl, and fixed with 4% paraformaldehyde. Fixed cell layers were stained with 0.5% alcian blue or 0.1% toluidine blue to examine chondrogenesis. For alkaline phosphatase, fixed cells were incubated with 0.125% β-naphthyl phosphate and 0.5% fast blue RR salt in 100 mM Tris buffer, pH 8.8, containing 5 mM $MgCl_2$. Mineral deposition was assessed by a modified von Kossa staining technique. After 30 min of incubation with 3% $AgNO_3$ in the dark, cell layers were exposed for 30 s to 254-nm ultraviolet light on a viewing box to rapidly develop the black silver phosphate nodules. To visualize cells undergoing DNA replication (S-phase), [^{3}H]thymidine-treated cultures were coated with Kodak NTB2 emulsion for autoradiography (Eastman Kodak Co., Rochester, NY) and counterstained with Giemsa after development.

Northern Analysis

For Northern analysis, cells were plated in 6-multiwell plates at a density of 3.6 × 10^5 cells per 2-ml/well and cultured for 4, 7, and 10 d in media containing 0, 4, and 40 ng OP-1/ml. Total RNA was extracted by the acid guanidine thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Briefly, after washing with cold PBS, the cells were homogenized in Solution D, which contained 4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% *N*-lauroyl-sarcosine, 10 mM *trans*-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA), and 100 mM β-mercaptoethanol, pH 7.15. One volume of water-saturated phenol, 0.2 volume of chloroform-isoamyl alcohol (49:1), and 0.1 volume of 2 M sodium acetate, pH 4.0, were added to the homogenate, and total RNA was extracted into the aqueous phase after centrifugation. Isopropanol-precipitated RNA was again solubilized in Solution D and reextracted by the same procedure to increase purity.

5-μg samples of total RNA were electrophoretically resolved in 1% formaldehyde agarose gels and transferred to nylon membrane filters (Nytran; Schleicher & Schuell, Keene, NH) by capillary blotting. Filter blots were prehybridized 4 h at 45°C with buffer containing 50% formamide, 5× SSPE, 5× Denhardt's solution, 1% SDS and 25 μg/ml of salmon sperm DNA. Hybridizations were carried out for 16 h at 45°C with buffer containing 50% formamide, 5× SSPE, 1% SDS, 10% dextran sulfate, 25 μg/ml of salmon sperm DNA, and the desired cDNA probe labeled with [α^{32}P]-dCTP by random priming (Kit; Boehringer Mannheim Corp.). Probes included the cDNA for rat α_1(I) collagen (Genovese et al., 1984), rat pro-α_1(II) collagen (Kohno et al., 1984), rat α_1(IX) collagen and rat α_1(X) collagen (Nishimura, I., unpublished observation), and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Sabath et al., 1990). After washing the filters, autoradiograms were made at −80°C by exposure to Kodak XAR-5 film (Eastman Kodak Co.).

Results

Cell Growth

Effects of OP-1 on cell proliferation were evaluated in two ways: [^{3}H]thymidine incorporation into acid-insoluble DNA, and total DNA content of cell layers. OP-1 stimulates DNA synthesis in the early stages of culture where cells are sparse and generally proliferating. A 120% stimulatory effect of OP-1 is significant at day 4, but there are no apparent differences in [^{3}H]thymidine incorporation between OP-1–treated cultures and control cultures after confluence (day 10–13) (Fig. 1 *B*). At day 7, [^{3}H]thymidine incorporation is increased 55 and 50% by 20 and 40 ng/ml of OP-1, respectively. OP-1 at 100 ng/ml does not significantly elevate labeling, in part because cultures with this high dose have reached confluence by this 7-d assay point (Fig. 1 *A*). These results on cell proliferation are confirmed by measurements of DNA content. Although OP-1 increases DNA content by 30–60% in a dose-dependent manner at day 7 (Fig. 1, *C* and *D*), significant differences in DNA content are not observed after day 10 (Fig. 1 *D*).

Chondrogenic Differentiation

Alcian blue staining of sulfated glycosaminoglycan was used to evaluate chondrogenesis, since the production of this extracellular matrix component is a hallmark of chondrogenesis (Hunter and Caplan, 1983). The intensity of alcian blue staining shows dose-dependent increase caused by OP-1 (Fig. 2 *A*). Induction of alcian blue–stained colonies in OP-1–treated cultures peaks at day 7, and the intensity of the staining decreases gradually at later times (Fig. 2 *B*). Clusters of round, refractile, alcian blue–stained cells appear within two days after treatment with OP-1 (day 3), and increase in both size and number until day 7. These cells then become enlarged and hypertrophic, and gradually disappear. Although the intensity of alcian blue staining in control cultures increases gradually by day 13 (Fig. 2 *B*), this is caused by diffuse binding of dye to the entire cell layer; histological examination shows total absence of alcian blue–stained colonies, in contrast to their abundance in OP-1–treated cultures.

Analysis of the dose dependence of the OP-1 induction of chondrocytic cell clusters was carried out by plating cells on day 0 in the presence of different concentrations of OP-1 (Fig. 2 *C*). On day 5, wells were scored for total numbers of "clonal" clusters containing three or more cells. Fig. 2 *C* shows that concentrations of 10 ng/ml OP-1 or greater induce a highly significant increase in the number of these clusters, with a fourfold increase over background at 10 ng/ml and a 50-fold increase at 320 ng/ml.

Northern analysis of mRNA levels was performed with collagen type I, II, IX, and X cDNAs to evaluate the chondrocytic properties of OP-1–induced cells. Type II collagen mRNA is increased by OP-1 treatment in a dose-dependent manner, while type I collagen message decreases slightly (Fig. 3, *I* and *II*). Interestingly, short form type IX collagen message is expressed even in control cultures, while long form type IX collagen message is expressed only in OP-1

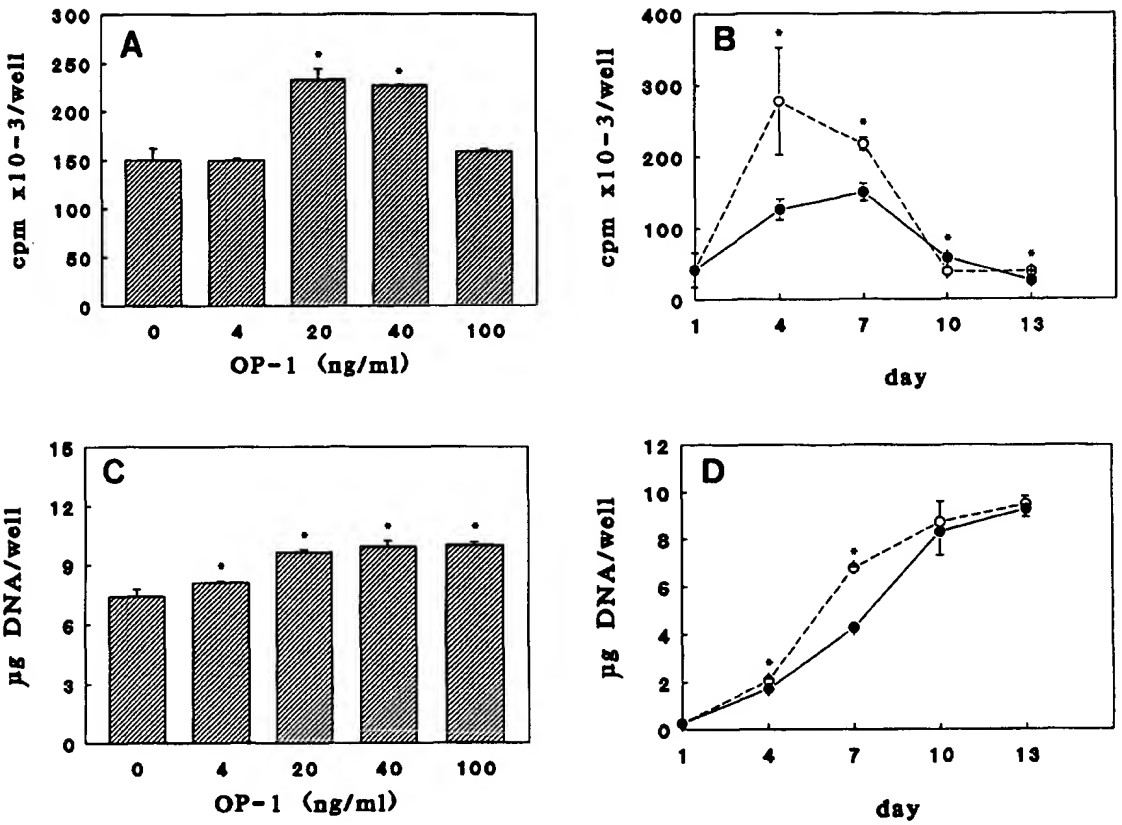

Figure 1. OP-1 effects on cell proliferation, showing the dose dependence (*A*) and time course (*B*) of [^{3}H]thymidine incorporation into acid-insoluble DNA, and the dose dependence (*C*) and time course (*D*) of changes in DNA content of cell layers. Cell layers were pulse labeled with [^{3}H]thymidine (2 µCi/ml, 4 h) before harvest (*A* and *B*). DNA was quantitated in cell layers (*C* and *D*) by the fluorometric diaminobenzoic acid method. For dose dependence, cells were cultured with graded concentrations of OP-1 for 6 d from day 1 and harvested at day 7. For kinetics, cells were cultured without OP-1 (•) or with 40 ng/ml OP-1 (○) from days 1–13, and harvested every 3 d beginning on day 1. Data represent the mean ± SD of three cultures for each treatment and are representative of three separate experiments. Asterisk, significant difference from control; $P \leq 0.05$.

treated cultures (Fig. 3, *IX*). Type II and IX collagen messages peak at day 4 and then gradually decrease. OP-1 induced the expression of type X collagen message only at day 7 in the culture treated with 40 ng/ml of OP-1 (Fig. 3, *X*).

Alkaline Phosphatase Activity

Alkaline phosphatase-specific activity is significantly increased by OP-1 in a dose-dependent manner; 3.4- and 4.5-fold at 40 and 100 ng/ml OP-1, respectively (Fig. 4 *A*). Activity in the OP-1 treated cultures peaks at day 7 and then decreases slightly (Fig. 4 *B*). In contrast, alkaline phosphatase in control cultures increases gradually throughout the culture period (Fig. 4 *B*).

Osteocalcin Production

Although the amount of osteocalcin production in early primary cultures (1–2 ng/µg DNA) is far less than in mature cultures, OP-1 decreases osteocalcin production in a dose-dependent manner at day 7 (Fig. 5 *A*). However, once the cultures have become confluent (days 10 and 13), the osteocalcin production becomes 10- to 20-fold greater than on day 7, and OP-1 significantly increases osteocalcin production (Fig. 5 *B*).

OP-1 Responsiveness of Different Calvarial Cell Populations

The histology of the developing rat calvaria features plates of membranous bone separated by fibrous sutures. At the outer limbs of the lambdoidal suture are remnants of the chondrocranium, from which Rifas et al. (1982) were able to derive chondrocytes by selective culture conditions. We examined the OP-1 response of cells from anatomically different regions of calvaria (frontal, parietal, and occipital bones, and sutures) (Table I). Cells from all regions showed OP-1–dependent increases of 1.5–3-fold in alcian blue staining, and 2.1–6-fold increases in alkaline phosphatase specific activity. It is noteworthy that untreated cultures from all regions have low alcian blue staining (Table I), indicating that few, if any, differentiated chondrocytes exist in any of these anatomical regions prior to culture in the presence of OP-1. However, the occipital and parietal populations appear to have a greater number of cells with OP-1–dependent chondrogenic potential.

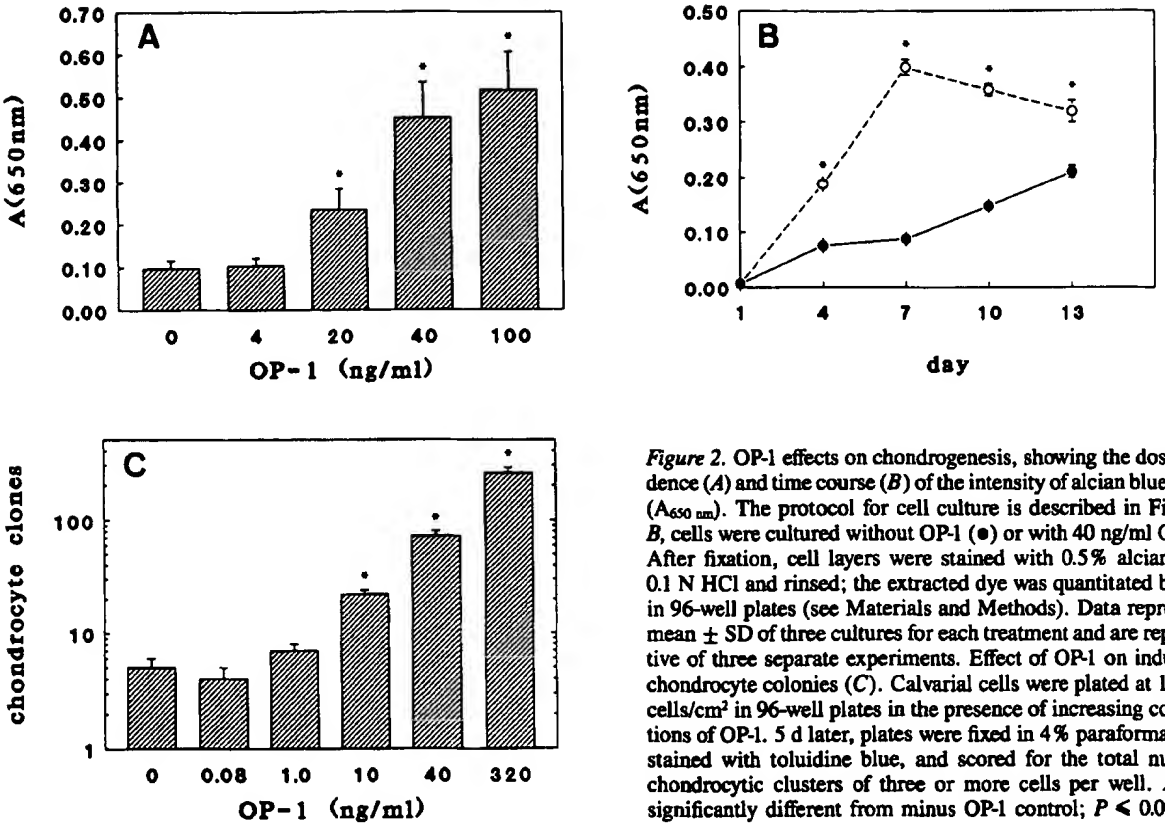

Figure 2. OP-1 effects on chondrogenesis, showing the dose dependence (*A*) and time course (*B*) of the intensity of alcian blue staining ($A_{650\,nm}$). The protocol for cell culture is described in Fig. 1; for *B*, cells were cultured without OP-1 (•) or with 40 ng/ml OP-1 (○). After fixation, cell layers were stained with 0.5% alcian blue in 0.1 N HCl and rinsed; the extracted dye was quantitated by $A_{650\,nm}$ in 96-well plates (see Materials and Methods). Data represent the mean ± SD of three cultures for each treatment and are representative of three separate experiments. Effect of OP-1 on induction of chondrocyte colonies (*C*). Calvarial cells were plated at 1.1×10^5 cells/cm² in 96-well plates in the presence of increasing concentrations of OP-1. 5 d later, plates were fixed in 4% paraformaldehyde, stained with toluidine blue, and scored for the total number of chondrocytic clusters of three or more cells per well. Asterisk, significantly different from minus OP-1 control; $P < 0.05$.

Sequential enzymatic digestion of calvarial bone segments in the standard protocol (frontal + parietal + occipital, trimmed free of sutures) releases cells with increased osteoblastic properties in later digests (Wong and Cohn, 1975). Similarly, we found that the chondrogenic potential increases significantly in later digests. OP-1 treatment (40 ng/ml, 6 d) causes increases in alcian blue staining of 1.66-fold in digest I cells, 2.54-fold in digest II, and 3.04-fold in pooled cells from digests III–V. The alkaline phosphatase in all three cell populations was elevated 5–6-fold by OP-1.

Effect of OP-1 on Osteoblastic Cell Populations of Different Age

Chondrogenic differentiation in this model system is observed only when primary cultures are treated early with OP-1 from day 0 (Fig. 2 *C*) or day 1 (Fig. 2, *A* and *B*; Table II). Quantitatively, alcian blue staining of the day 1–7 cultures increases 3.9-fold in response to OP-1, while the same duration of OP-1 treatment of confluent day 7–13 or subcultivated cultures causes little chondroinduction (Table II). Although the intensity of alcian blue dye uptake (A_{650nm}) by day 7–13 and subcultivated cultures with or without OP-1 exhibits a relatively high background, no alcian blue–stained cartilage-like clusters are observed histologically. Alkaline phosphatase specific activity, a marker for chondrocytes and osteoblasts, is also enhanced to a greater magnitude by OP-1 in primary cultures treated with OP-1 from day 1 (3.21-fold) than in more mature day 7–13 cultures or subcultivated cultures (Table II). However, the mature osteoblast marker os-

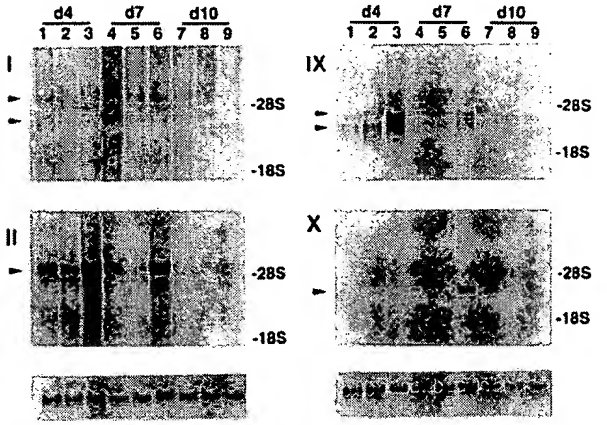

Figure 3. Northern analysis of gene expression, showing the effects of OP-1 on mRNA synthesis of collagen type I (*I*), type II (*II*), type IX (*IX*), and type X (*X*). Cells were cultured for 3 d (lanes *1*, *2*, and *3*), 6 d (lanes *4*, *5*, and *6*), and 9 d (lanes *7*, *8*, and *9*), with OP-1 treatment at 0 ng/ml (lanes *1*, *4*, and *7*), 4 ng/ml (lanes *2*, *5*, and *8*), and 40 ng/ml (lanes *3*, *6*, and *9*) beginning on day 1. Total RNA was extracted from each culture and Northern analysis was performed as described. Equivalent loading of total RNA (5 μg/lane) was verified by UV absorbance, ethidium bromide staining, and hybridization with a mouse GAPDH cDNA probe (*bottom panels*). For the analysis of types I and II collagen, and types IX and X collagen, the same filters were used. Arrows mark the collagen gene transcript size(s) for each collagen type detected by the specific cDNA probe. The positions of 28 S and 18 S ribosomal RNA are also indicated.

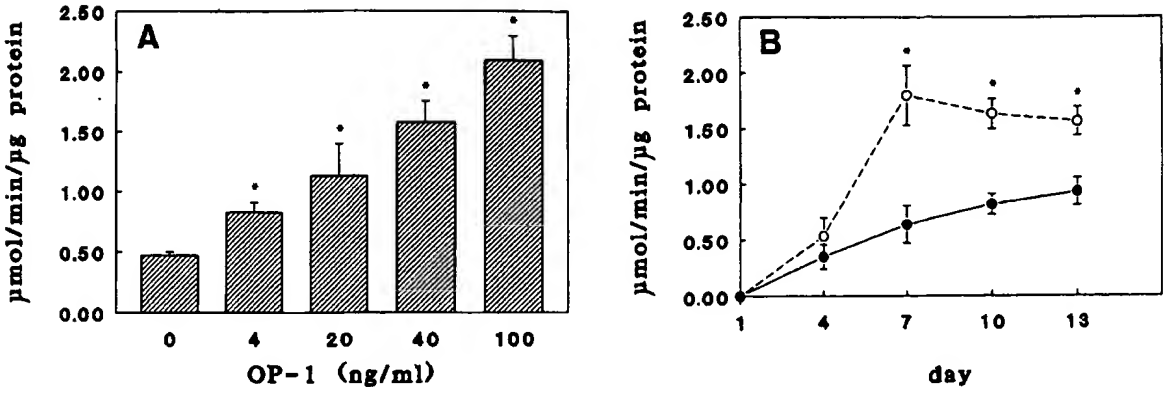

Figure 4. OP-1 effects on alkaline phosphatase specific activity, showing dose dependence (*A*) and time course (*B*) of the changes. The experimental protocol is described in Fig. 1; for *B*, cells were cultured without OP-1 (•) or with 40 ng/ml OP-1 (○). Alkaline phosphatase was measured spectrophotometrically in detergent-solubilized cell layers with *p*-nitrophenyl phosphate as a substrate. Enzyme specific activity is presented as μmol *p*-nitrophenol produced/min/μg protein. Data represent the mean ± SD of three cultures for each treatment and are representative of three separate experiments. Asterisk, significant difference from control; $P \leq 0.05$.

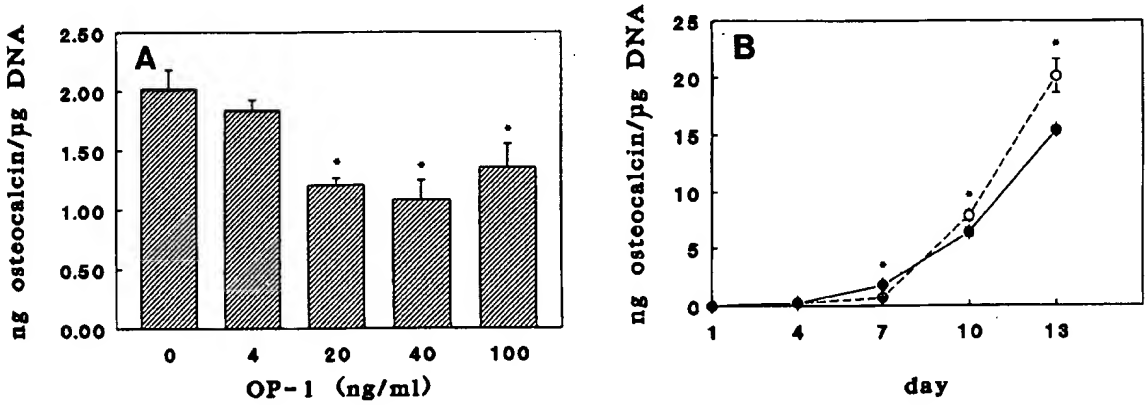

Figure 5. OP-1 effects on osteocalcin production, showing dose dependence (*A*) and time course (*B*) of osteocalcin levels in 3-d conditioned medium. The experimental protocol is described in Fig. 1; for *B*, cells were cultured without OP-1 (•) or with 40 ng/ml OP-1 (○). Osteocalcin was measured by specific radioimmunoassay. Data are expressed as total ng rat osteocalcin/μg cell layer DNA. Data represent the mean ±SD of three cultures for each treatment and are representative of three separate experiments. Asterisk, significant difference from control; $P \leq 0.05$.

Table I. OP-1 Effects on Different Calvarial Cell Populations

Calvarial region	OP-1	Alcian blue staining		Alkaline phosphatase	
		A(650) nm)	*T/C*	*μmol/min/μg protein*	*T/C*
Frontal	−	0.117 ± .010		0.279 ± .048	
	+	0.186 ± .007	1.59	0.709 ± .048	2.54
Parietal	−	0.190 ± .002		0.437 ± .025	
	+	0.503 ± .046	2.65	1.299 ± .168	2.97
Occipital	−	0.177 ± .018		0.121 ± .014	
	+	0.525 ± .033	2.97	0.731 ± .127	6.04
Suture	−	0.181 ± .006		0.579 ± .048	
	+	0.265 ± .014	1.46	1.216 ± .021	2.10

Calvarial regions were microdissected into pools containing anatomically defined regions (frontal bone, parietal bone, etc.). Cells from each region were enzymatically dissociated and plated at equal density on day 0 (see Materials and Methods); OP-1 treatment (40 ng/ml) was from days 1–7. Data represent mean ±SD from three replicate wells and are representative of several independent experiments. All ratios of OP-1 treated to control (*T/C*) showed significant OP-1 effects ($P \leq 0.05$).

teocalcin is decreased by OP-1 in primary cultures treated from day 1, but increased in subcultivated cell cultures (Table II). Thus, early treatment (days 1–7) with OP-1 elicits the greatest chondrogenic response (alcian blue, alkaline phosphatase) and a diminished osteogenic response (osteocalcin); late OP-1 treatment elicits minimal chondrogenic response and an enhanced osteoblastic response.

Effect of Ascorbic Acid and β-Glycerophosphate

OP-1 treatment in the absence of ascorbic acid increases chondrogenesis and alkaline phosphatase activity slightly, but these phenotypic properties are greatly increased when OP-1 is used in the presence of ascorbic acid (Table III). β-Glycerophosphate has no significant effect on the induction of chondrogenesis and alkaline phosphatase activity by OP-1 (Table III). Osteocalcin production is decreased by early OP-1 treatment (Table III; also Fig. 5 and Table IV). Although this OP-1–dependent decrease still occurs in the presence of ascorbate ± β-glycerophosphate, the addition of

Table II. OP-1 Effects on Osteoblastic Cell Populations of Different Age

Cell population	OP-1	Alcian blue staining		Alkaline phosphatase activity		Osteocalcin	
		A(650 nm)	*T/C*	*μmol/min/μg protein*	*T/C*	*ng/μg DNA*	*T/C*
Days 1–7	−	0.087 ± .005		0.490 ± .033		1.77 ± 0.07	
	+	0.339 ± .014	3.90*	1.573 ± .101	3.21*	0.68 ± 0.10	0.38*
Days 7–13	−	0.245 ± .015		0.637 ± .028		4.35 ± 0.34	
	+	0.289 ± .005	1.18*	0.785 ± .067	1.23*	4.97 ± 0.80	1.14
Subcult.	−	0.202 ± .009		0.560 ± .056		4.14 ± 0.26	
	+	0.212 ± .020	1.05	1.186 ± .031	2.12*	7.70 ± 1.54	1.86*

Cell cultures were established (see Materials and Methods) and treated with or without 40 ng/ml OP-1 for 6 d before analysis. *Days 1–7* indicates early treatment of subconfluent cells initiated on day 1 (cells were plated on day 0). *Days 7–13* indicates late treatment after cells have reached confluence. *Subcult.* indicates treatment of subcultivated cells which was initiated the day after trypsinization and replating of a day 7 culture. Data represent means ± SD from three replicate wells and are representative of several independent experiments.
* Ratios of OP-1 treated to control (*T/C*) which showed significant OP-1 effects ($P \leq 0.05$).

Table III. Dependence of OP-1 Effects on Ascorbate and β-Glycerophosphate (βGP)

Treatment group	OP-1	Alcian blue staining		Alkaline phosphatase activity		Osteocalcin	
		A(650 nm)	*T/C*	*μmol/min/μg protein*	*T/C*	*ng/ml*	*T/C*
Control	−	0.038 ± .003		0.011 ± .002		0.032 ± .043	
	+	0.048 ± .002	1.26	0.022 ± .003	2.00	0.007 ± .011	0.22
+Ascorbate	−	0.086 ± .002		0.053 ± .005		9.48 ± .25	
	+	0.276 ± .021	3.21*	0.204 ± .036	3.85*	6.57 ± .41	0.69*
+Asc +βGP	−	0.087 ± .002		0.053 ± .011		13.34 ± .65	
	+	0.316 ± .013	3.63*	0.201 ± .031	3.79*	9.66 ± 1.75	0.72*

Cell cultures were established on day 0 and treated from days 1–7 with ascorbate alone (50 μg/ml), or with ascorbate + β-glycerophosphate (10 mM), or without these additives (*Control*). These three groups were also treated with 40 ng/ml OP-1 (+) or vehicle (−) from days 1–7 before analysis. Data represent means ± SD from three replicate wells and are representative of several independent experiments.
* Ratios of OP-1 treated to control (*T/C*) which showed significant OP-1 effects ($P \leq 0.05$).

β-glycerophosphate elevates basal osteocalcin production compared to ascorbate alone (Table III).

Morphological and Histochemical Changes Induced by OP-1

The biochemical evidence for OP-1-dependent chondroinduction is consistent with histological evaluations. Cultures treated with OP-1 have abundant clusters of round cells which are stained with alcian blue intensively (Fig. 6 *B*). In contrast, there are no alcian blue-stained colonies in control cultures, while small colonies of tightly compacted, flattened cells are observed (Fig. 6 *A*). Staining of OP-1 treated cultures with toluidine blue shows red/purple metachromasia of these colonies (Fig. 6 *H*). Histochemically, strong alkaline phosphatase staining is observed for both the large, round chondrocyte-like cells and the small, compact cells surrounding these chondrocytes in OP-1-treated cultures (Fig. 6 *D*). In control cultures, the chondrocytic colonies are absent and alkaline phosphatase is restricted to tightly compacted cell colonies (Fig. 6 *C*). Mineralization of primary cultures occurs in the day 7–13 period only if 10 mM β-glycerophosphate is added to the medium. von Kossa staining shows strong mineralization in OP-1-treated cultures. Extensive mineralization occurs on the periphery of each large round cell in the chondrocyte-like cell clusters in OP-1-treated cultures (Fig. 6 *F*), while only a small amount of mineralization is observed in control cultures at day 7 (Fig. 6 *E*). Autoradiograms of [^{3}H]thymidine-labeled cultures at day 4 show that small cells surrounding the clusters of large round chondrocytic cells incorporate [^{3}H]thymidine prosperously, while the large cells within the colonies do not (Fig. 6 *G*).

TGF-β1 Antagonism of OP-1 Effects

Because OP-1 is a member of the TGF-β superfamily of growth and differentiation factors, comparative studies of OP-1 and TGF-β1 were initiated in this chondroinduction model system. These two factors share 34% identity in their 7-cysteine domain (Ozkaynak et al., 1990). Histological (Fig. 7) and biochemical (Table IV) changes associated with OP-1-dependent chondrogenesis are not seen with TGF-β1 over a dose range of 0.01–40 ng/ml. Furthermore, the chondrogenesis induced by 40 ng/ml of OP-1 alone (4.84-fold increase in alcian blue, 1.47-fold increase in alkaline phosphatase; Table IV) is strongly antagonized by TGF-β1 concentrations of 1 and 10 ng/ml (Fig. 7; Table IV). TGF-β1 also diminishes osteoblastic parameters osteocalcin and alkaline phosphatase (Table IV). Histologically, TGF-β1 changed the normal polygonal cell shape to a fibroblastic spindle shape (Fig. 7).

Discussion

Evidence That OP-1 Induces Chondrogenesis In Vitro

We present evidence that OP-1 induces chondrogenesis in a primary culture model system initiated from newborn rat calvaria. Morphologically, chondrocytic colonies appear within two days after treatment with OP-1, and these distinc-

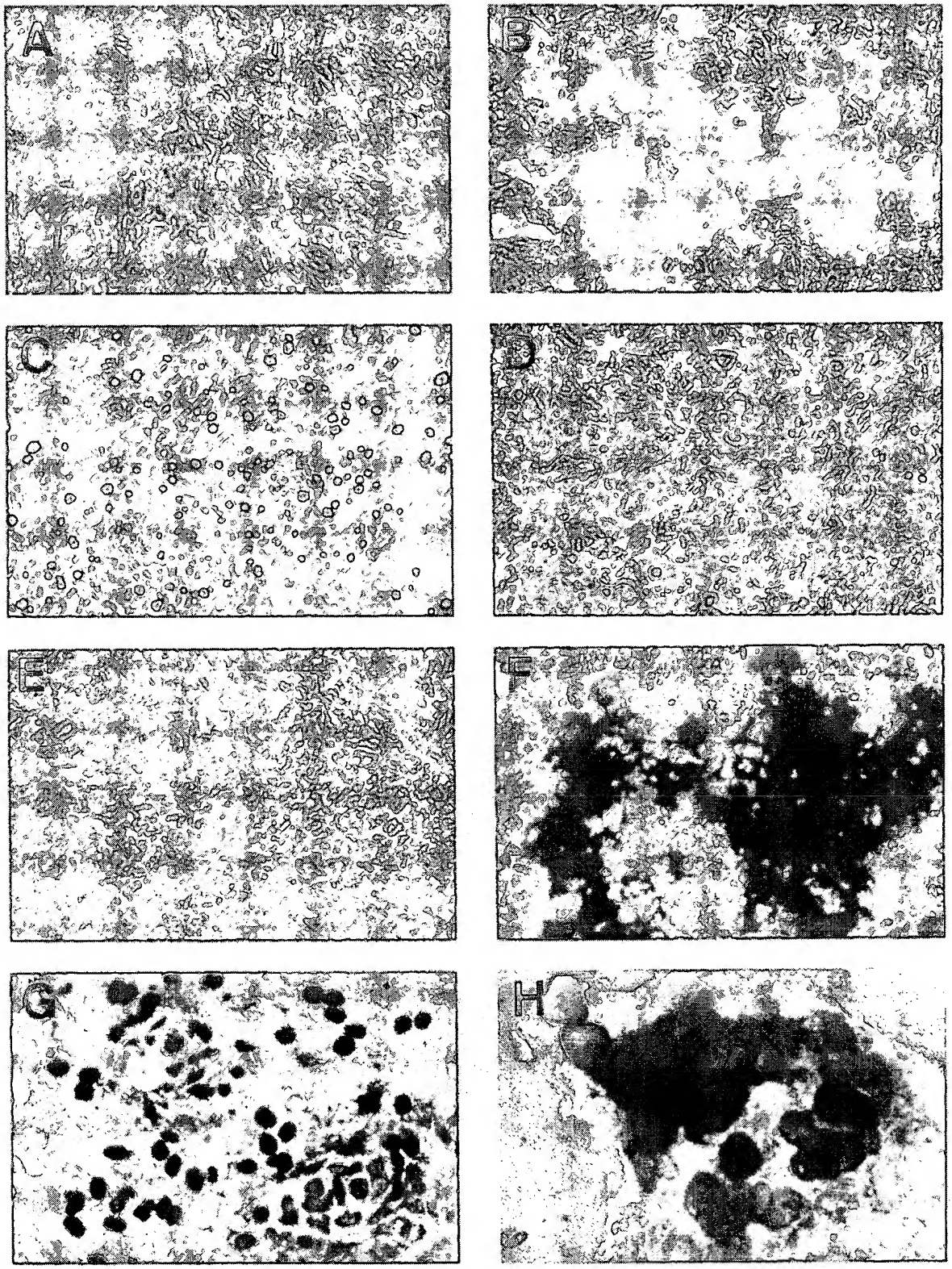

Figure 6. Histological changes induced by OP-1. Phase contrast micrographs of alcian blue staining (*A* and *B*), alkaline phosphatase staining (*C* and *D*), and von Kossa staining (*E* and *F*); autoradiogram of [^{3}H]thymidine incorporation into DNA (*G*); light micrograph of toluidine blue staining (*H*). Cells were cultured without OP-1 (*A*, *C*, and *E*) or with 40 ng/ml of OP-1 (*B*, *D*, *F*, and *H*) for 6 d from day 1, and the effects of OP-1 on chondrogenesis (*A*, *B*, and *H*) alkaline phosphatase activity (*C* and *D*) and mineralization (*E* and *F*) were examined at day 7. For autoradiography (*G*), cells were cultured with 40 ng/ml of OP-1 for 3 d from day 1, pulsed with [^{3}H]thymidine for 4 h, and autoradiographed.

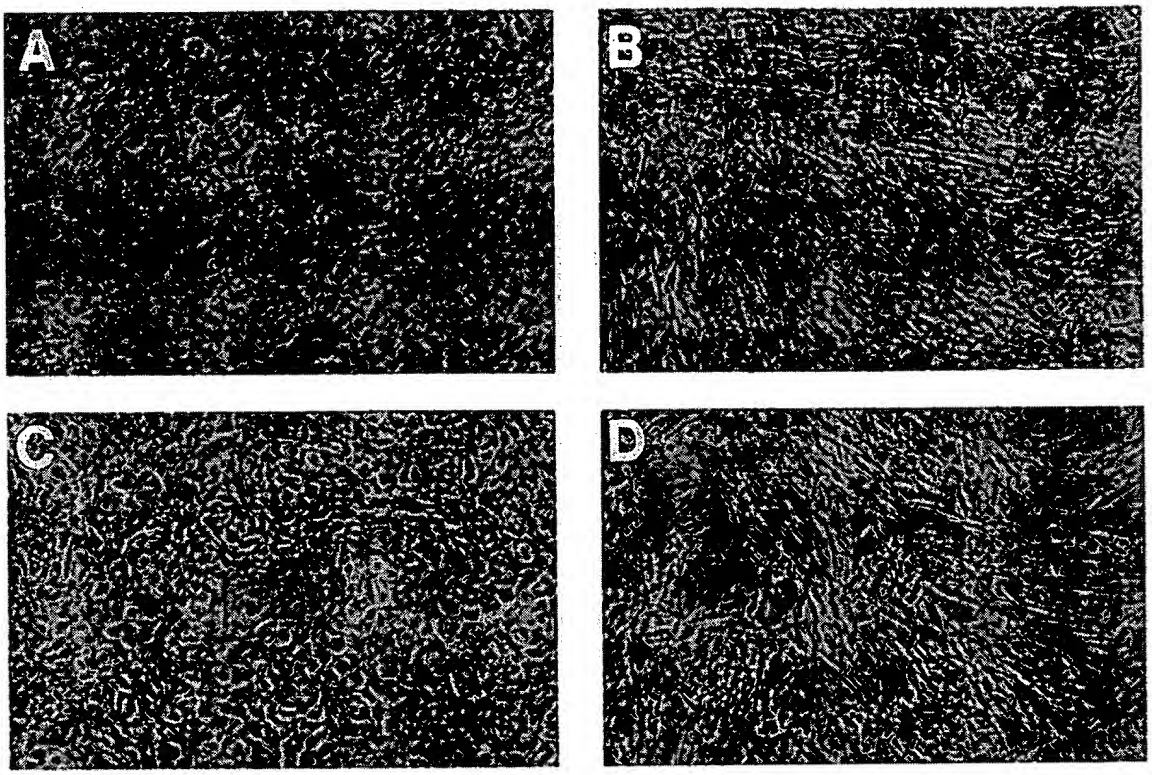

Figure 7. TGF-β1 antagonism of OP-1 chondroinduction. Phase-contrast micrographs are shown for the control culture (*A*), and cultures treated with either 1 ng/ml TGF-β1 (*B*), 40 ng/ml OP-1 (*C*), or 1 ng/ml TGF-β1 and 40 ng/ml OP-1 (*D*) for 6 d from day 1.

tive cell clusters increase in both number and size before becoming hypertrophied. Chondrocytic cell clusters stain heavily with alcian blue, stain metachromatically with toluidine blue, express high alkaline phosphatase activity, and act as loci for the initiation of mineralization which first occurs in and around the hypertrophic chondrocytic cells. These sequential changes have strong parallels to the process of endochondral bone formation in vivo, as discussed below.

Table IV. TGF-β1 Antagonism of OP-1 Induced Phenotypic Changes

Treatment		Ratio of treated/control		
TGF-β1	OP-1	Alcian blue staining	Alkaline phosphatase activity	Osteocalcin
ng/ml				
–	–	1.00	1.000	1.000
0.1	–	0.98	0.367*	0.094*
1	–	0.99	0.083*	0.056*
1	40	1.41*	0.250*	0.055*
10	–	1.09	0.058*	0.062*
10	40	1.43*	0.215*	0.059*
–	40	4.84*	1.465*	0.242*

Cell cultures were established (see Materials and Methods) and treated with or without various doses of TGF-β1 and/or OP-1 for 6 d (culture day 1–7) before analysis. Data were calculated from means of three replicate wells; standard deviations are ±5% for alcian blue staining, and ±12% for alkaline phosphatase activity and osteocalcin.

* Ratios of treated to control values which showed significant differences from the untreated control ($P \leq 0.05$).

Expression of cartilage-associated type II and IX collagen genes is enhanced in the early stages after exposure to OP-1, and then gradually decreases. It is noteworthy that long-form type IX collagen is expressed only in cultures treated with OP-1, while type II and short-form type IX collagens are expressed even in control cultures. Although the association of type II and IX collagens with chondrogenesis is well established (von der Mark, 1980; Heinegard and Paulsson, 1987), type II collagen is also expressed in a variety of other tissues during embryogenesis, thus it is not a definitive marker for the chondrocyte phenotype (Chah et al., 1991; Kosher and Solursh, 1989). Type IX collagen can be synthesized in two distinct forms due to differential mRNA transcription of the α1(IX) gene, using alternative promotor/transcriptional start sites (Nishimura et al., 1989). In cartilage, the upstream promoter/transcriptional start site is predominantly activated, resulting in processing of the long form containing the 5′ exons encoding the NC4 globular amino-terminal domain. It has been demonstrated that the downstream promoter/transcriptional start site (short form α1[IX] mRNA) is predominantly used in the nonchrondrogenic tissues, including chicken primary cornea (Svoboda et al., 1988; Nishimura et al., 1989), nonchondrogenic regions of developing chicken forelimb (Swiderski and Solursh, 1992*a*), and notochord (Swiderski and Solursh, 1992*b*). The OP-1–dependent appearance of long-form type IX collagen strongly supports our inference that OP-1 induces chondrogenesis.

Type X collagen is well known to be a specific molecule of hypertrophic cartilage (Linsenmayer et al., 1988), and it

appears with the onset of hypertrophic cartilage mineralization (Linsenmayer et al., 1991). While expression of both type II and IX collagen genes gradually diminishes through the culture period, type X collagen mRNA appears only transiently after 6 d of OP-1 treatment (Fig. 3). Thus it appears that OP-1 is capable not only of inducing chondrogenesis, but also of promoting the further differentiation of chondrocytes to a hypertrophic state analogous to that which occurs in the growth plates of long bones.

OP-1–dependent Chondrogenic Potential of Calvarial Cells

Development of the calvaria follows the classical process of intramembranous ossification. Osteoblasts are derived from mesenchymal fibroblast-like precursors, or osteoprogenitor cells. Osteogenesis proceeds without histological evidence of cartilage involvement. However, there are elements of the embryonic chondrocranium associated with the distal regions of the lambdoidal suture and small portions of the adjacent parietal and occipital bones. Rifas et al. (1982) showed that floating cells derived from ascorbate-free cultures of the calvarial chondrocranium could redifferentiate into chondrocytes and form cartilage nodules in the presence of ascorbate. To rule out the possibility that early OP-1 treatment was acting only on cells derived from the chondrocranium, calvarial segments were dissected and studied separately (Table I). It is clear that alcian blue–stained clusters of hypertrophic chondrocytes can form from cells derived from all calvarial regions, although they are somewhat more abundant in the occipital and parietal bones. Another conclusion from sequential calvarial digestion studies is that the greatest abundance of cells with OP-1–dependent chondrogenic potential occurs in digests III–V, cells indigenous to the mineralizing osseous matrix which are also known to have the greatest osteogenic potential. In contrast, cells from calvarial digest populations I and II, which are depleted of osteoprogenitor cells, show a poor chondrogenic response to OP-1. This supports the hypothesis that pluripotent osteoprogenitor cells are the target cells for OP-1–dependent chondroinduction.

In the early stages of the cultures, OP-1 stimulates DNA synthesis. Because chondrogenic differentiation is also stimulated in this early period, there is an apparent contradiction to the general rule of an inverse relationship between cell proliferation and differentiation. However, the autoradiogram of [^{3}H]thymidine incorporation into DNA at day 4 shows that the clusters of large round cells which seem to be incipient chondrocytes induced by OP-1 do not incorporate [^{3}H]thymidine, while small cells surrounding the colonies do so intensively (Fig. 6 *G*). It is possible that OP-1 stimulates DNA synthesis in undifferentiated progenitor cells, but is a weaker mitogen for differentiated cells. Judging from the typical hypertrophic chondrocyte cluster size of 2–20 cells, OP-1 allows ~1–4 rounds of cell division in cells responding to the chondroinductive influence.

Ascorbic acid is essential for the induction of chondrogenesis by OP-1. In rat primary osteoblast-like cell cultures, ascorbate is known to be required for expression of the osteoblastic phenotype (Aronow et al., 1990), and ascorbate induces alkaline phosphatase and mineralization in chick chondrocyte cultures (Leboy et al., 1989). Though the precise role of this vitamin in chondrogenesis and osteogenesis is still unclear, ascorbic acid is required for the hydroxylation of proline and lysine residues and promotes collagen secretion (Kivirikko and Myllyla, 1987). In a recent study of MC3T3-E1 osteoblasts, Franceschi and Iyer (1992) showed that expression of mature osteoblastic markers is reversibly blocked when collagen synthesis and secretion is inhibited by ascorbate deprivation or addition of proline analogs. It is likely that the existence of a properly assembled, mature collagenous extracellular matrix plays a critical role in the induction of chondrogenesis and osteogenesis.

OP-1 Regulates the Commitment of Osteoprogenitor Cells

Primary cultures prepared through sequential collagenase digestions of fetal or newborn rat calvaria have long been known to possess osteoblastic characteristics (Wong and Cohn, 1975; Bellows et al., 1986). A temporal appearance of osteoblastic phenotypic markers in these in vitro systems is analogous to that seen in vivo in developing fetal rat calvaria tissue (Aronow et al., 1990). A similar correlation of temporal expression exists for embryonic chicken osteoblasts (Gerstenfeld et al., 1987). Owen et al. (1990) proposed a model wherein primary rat calvarial cell cultures were considered to traverse three principal periods of osteoblast development, consisting of proliferation, matrix development/maturation, and mineralization. They presented evidence that proliferation was downregulated before the onset of events associated with osteoblast differentiation.

The rat calvarial cell culture system which we have used expresses osteoblastic characteristics after confluence, and this osteogenesis is known to be amplified by OP-1 treatment after confluence. We reported previously that OP-1 stimulates osteoblastic properties such as alkaline phosphatase activity, intracellular cAMP production in response to parathyroid hormone (PTH), osteocalcin synthesis, and mineralization (Sampath et al., 1992).

Chondroinductive effects of OP-1, the focus of this study, are observed in rat calvarial cell primary cultures when exposure occurs early, during the proliferation phase before confluence. It is noteworthy that early OP-1 treatment decreases osteocalcin production when the level of osteocalcin synthesis is relatively low (Fig. 5 and Tables II and IV). This suggests that OP-1 delays the osteoblastic differentiation of uncommitted osteoprogenitor cells, either by stimulating the proliferation of this subpopulation, or by diverting cells to a chondrogenic pathway. As the culture matures in the absence of added OP-1, these progenitors either die or become increasingly committed to the osteoblastic lineage, since later exposure to OP-1 promotes further maturation and expression of osteoblastic phenotypes by acting on the predominant target population of committed preosteoblasts and osteoblasts in mature cultures (Sampath et al., 1992). Reversal of these committed cells by OP-1 from osteogenic to chondrogenic fate apparently does not occur, and there is no obvious formation of chondrocytic clusters during late OP-1 treatment. Table II also supports this inference by demonstrating OP-1 enhancement of osteoblastic, but not chondrocytic, markers in subcultivated cells.

We have observed similar OP-1 effects on ROS 17/2.8 cells and MC3T3-E1 cells (unpublished data): OP-1 enhances both alkaline phosphatase activity and osteocalcin produc-

tion in these osteoblastic cell lines. These effects of OP-1 on mature osteoblasts are consistent with the studies using related TGF-β superfamily proteins BMP-2, BMP-4, and BMP-3 (osteogenin) (Chen et al., 1991; Takuwa et al., 1991; Vukicevic et al., 1989). In the calvarial cell primary culture model, the chondrogenic versus osteogenic effects of OP-1 are, therefore, primarily determined by the timing of the onset of OP-1 treatment.

The exact lineage of cells which differentiate into chondrocytes under the influence of OP-1 is not yet known. The primary rat calvarial cell population used in this study (digest III-V) is dominated by osteoprogenitor cells, preosteoblasts, and osteoblasts (Wong and Cohn, 1975; Bellows et al., 1986; Bellows and Aubin, 1989; Aronow et al., 1990). Clonal cell lines which have characteristics of immature versus mature stages of osteoblastic differentiation and developmental commitment have been derived from rat calvaria (Yamaguchi and Kahn, 1991). Another clonally selected rat calvarial cell line was found to express the chondrocytic phenotype (Bernier et al., 1990). Other evidence exists that a clonal cell line derived from fetal rat calvaria can differentiate into several cell types. RCJ3.1, clonally derived from the 21-d fetal rat calvaria, differentiates into four distinct phenotypes including myoblasts, mineralized osteoblasts, adipocytes, and chondrocytes, the latter after dexamethasone treatment (Grigoriadis et al., 1988). Kellerman et al. (1990) demonstrated that an SV-40 immortalized cell line derived from mouse teratocarcinoma differentiates into osteoblasts, chondrocytes, and adipocytes under various culture conditions. It has also been shown that chick periosteal cells (Nakahara et al., 1992), bone marrow cells (Berry et al., 1992), and calvarial cells (Manduca et al., 1992) contain osteoprogenitor cells that are capable of differentiating into osteoblasts or chondrocytes.

The hypothesis that early OP-1 treatment targets uncommitted osteoprogenitor cells is supported by our findings, but we cannot rule out the possibility that OP-1 stimulates cartilage phenotypic expression among cells already committed to the chondrocytic lineage. The frequency of hypertrophic chondrocyte colonies induced by OP-1 is ~0.7% of plated cells in our experiments. Bellows and Aubin (1989) determined an osteoprogenitor cell frequency of 0.3% in the rat calvarial cell culture system by limited dilution counting of mineralized nodules.

TGF-β1 appears to strongly influence osteoprogenitor cell commitment in this primary culture model. The phenotypic changes (Table IV) and the fibroblastic appearance of the TGF-β1–treated calvarial cells, even in the presence of 40 ng/ml OP-1 (Fig. 7, *B* and *D*), suggests that TGF-β1 causes diversion of the uncommitted osteoprogenitor cells from both chondrogenic and osteogenic fates. This contrasts with reported TGF-β1 effects in vivo where subperiosteal injections of 200 ng/day in the rat femur cause elevated chondrogenesis (Joyce et al., 1990).

OP-1 Treatment In Vitro as a Model for Endochondral Ossification

In primary calvarial cultures, OP-1 induces chondrogenesis and fosters further differentiation of chondrocytes to a hypertrophic state. These centers of hypertrophic chondrocytes which develop in response to OP-1 in vitro appear to "nucleate" and organize local osteogenesis and mineralized nodule development (Fig. 6), just as calcified cartilage in the endochondral growth plate acts as a substratum for osteoblastic bone formation in vivo.

Differentiated chondrocytes are known to produce growth factors such as basic FGF which may locally stimulate osteoblast proliferation (Hauschka et al., 1986). A heparan- and chondroitin-SO_4-rich proteoglycan, betaglycan, is the class III TGF-β receptor (Cheifetz et al., 1987) and in a soluble, mobile form, betaglycan has been implicated in TGF-β binding to the extracellular matrix (Andres et al., 1989). Might proteoglycans such as betaglycan, decorin (DS-PGII), and biglycan (DS-PGI) play a role in tethering OP-1 to the matrix surrounding hypertrophic chondrocytes, thereby allowing a localized osteogenic response?

We have presented evidence in support of the hypothesis that OP-1 induces the differentiation of chondrocytes from uncommitted osteoprogenitor cells. It is possible that OP-1 is naturally involved in the induction of chondrocytes from these progenitors, thus playing an essential role in normal developmental and tissue repair processes of the skeletal system in vivo.

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Recombinant Human Bone Morphogenetic Protein-2 Stimulates Osteoblastic Maturation and Inhibits Myogenic Differentiation In Vitro

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Abstract. The in vitro effect of recombinant human bone morphogenetic protein-2 (rhBMP-2) on osteogenic and myogenic differentiation was examined in two clonal cell lines of rat osteoblast-like cells at different differentiation stages, ROB-C26 (C26) and ROB-C20 (C20). The C26 is a potential osteoblast precursor cell line that is also capable of differentiating into muscle cells and adipocytes; the C20 is a more differentiated osteoblastic cell line. Proliferation was stimulated by rhBMP-2 in C26 cells, but inhibited in C20 cells. rhBMP-2 greatly increased alkaline phosphate (ALP) activity in C26 cells, but not in C20 cells. The steady-state level of ALP mRNA was also increased by rhBMP-2 in C26 cells, but not in C20 cells. Production of 3',5'-cAMP in response to parathyroid hormone (PTH) was dose-dependently enhanced by adding rhBMP-2 in both C26 and C20 cells, though the stimulatory effect was much greater in the former. There was neither basal expression of osteocalcin mRNA nor its protein synthesis in C26 cells, but they were strikingly induced by rhBMP-2 in the presence of 1α,25-dihydroxyvitamin D_3. rhBMP-2 induced no appreciable changes in procollagen mRNA levels of type I and type III in the two cell lines. Differentiation of C26 cells into myotubes was greatly inhibited by adding rhBMP-2. The inhibitory effect of rhBMP-2 on myogenic differentiation was also observed in clonal rat skeletal myoblasts (L6). Like BMP-2, TGF-β1 inhibited myogenic differentiation. However, unlike BMP-2, TGF-β1 decreased ALP activity in both C26 and C20 cells. TGF-β1 induced neither PTH responsiveness nor osteocalcin production in C26 cells, but it increased PTH responsiveness in C20 cells. These results clearly indicate that rhBMP-2 is involved, at least in vitro, not only in inducing differentiation of osteoblast precursor cells into more mature osteoblast-like cells, but also in inhibiting myogenic differentiation.

Ectopic bone formation is elicited at intramuscular sites by implantation of bone inducing factors contained in demineralized bone matrix (28, 35, 36). This indicates that cells of the osteoblast lineage have a close relationship with those of the muscular lineage in their ontogeny, and the development of the two cell lineages may be mutually regulated by some factor(s) stored in bone matrix. The components and the action mechanism of these bone inducing factors had long remained obscure. This is mainly due to the difficulties of purification of these factors and the lack of suitable in vitro bioassay systems.

Recently, a number of laboratories have isolated bioactive proteins which induce cartilage and/or bone formation at the sites implanted (1, 13, 30, 39, 40). Human cDNAs for seven different bone morphogenetic proteins (BMPs),[1] BMP-1, BMP-2 (BMP-2A[41]), BMP-3 (osteogenin [13]), BMP-4 (BMP-2B[41]), BMP-5, BMP-6 (Vgr-1 [14]), and BMP-7 (OP-1[25, 30]) have been cloned (41, 42). The sequences deduced from these cDNAs have indicated that BMP-2 through BMP-7 are members of transforming growth factor-β (TGF-β) superfamily (14, 25, 41, 42). Furthermore, active recombinant human bone morphogenetic protein-2 (rhBMP-2) has been produced, which formed bone tissue in vivo when it was implanted (40). Although several bone-inducing factors were purified from bone matrix, the direct action of the isolated proteins on the osteoblastic cell lineage has not been fully investigated, except for osteogenin (37, 38). Furthermore, the biological action of the factors on nonskeletal tissues has not been extensively explored; since their biological activity has been evaluated only by their in vivo implantation at ectopic sites (1, 13, 30, 39, 40, 41).

To understand more precisely the mechanism of actions of these bone-inducing factors, a suitable in vitro experimental model is required. We recently established and character-

1. *Abbreviations used in this paper*: ALP, alkaline phosphatase; BMP, bone morphogenetic protein; PTH, parathyroid hormone; rh, recombinant human; TGF-β, transforming growth factor-β.

ized five rat clonal osteoblastic cell lines from neonatal rat calvariae (44). These cell lines express various osteoblastic properties, reflecting different maturation stages. Of these, two cell lines, ROB-C20 (C20) and ROB-C26 (C26), exhibit the extremes in maturation stages of osteoblasts. The C20 expresses a wide spectrum of osteoblastic properties, indicating that it is a mature osteoblastic cell line (44). In contrast, the C26 has characteristics of an osteoblast progenitor cell line, retaining the potential to differentiate into both myotubes and adipocytes (44). But neither of these cells produced osteocalcin (bone Gla protein, BGP), a bone matrix protein specifically produced by osteoblasts (11). C20 and C26 cells thus may be useful tools for determining the role of BMPs in osteogenic and myogenic differentiation.

Here we show that the two cell lines, C20 and C26, are responsive to rhBMP-2: this recombinant cytokine not only stimulated proliferation and differentiation of C26 cells, but inhibited myogenic differentiation of C26 cells in vitro. We also demonstrate that rhBMP-2 strikingly induces the synthesis of osteocalcin in the presence of 1α, 25-dihydroxyvitamin D_3 in C26 cells.

Materials and Methods

Recombinant Human Bone Morphogenetic Protein-2 and Transforming Growth Factor-β1

Recombinant human bone morphogenetic protein-2 (rhBMP-2) was produced by CHO (Chinese hamster ovary) cells and purified as described previously (40). The recombinant protein used in the present study was 80-90% pure as judged by silver-stained bands on SDS gels and also by NH_2-terminal sequencing. The in vivo ectopic bone formation assay revealed that this material was active at 1 μg, the lowest dose level tested 7 d after implantation.

TGF-β1 purified from human platelets was purchased from R & D Systems, Inc. (Minneapolis, MN).

Cell Culture

Two clonal rat osteoblastic cell lines, ROB-C20 (C20) and ROB-C26 (C26), were isolated from newborn rat calvaria (44). The two cell lines were plated into 24-multiwell plates (Falcon Labware, Lincoln Park, NJ) at a seeding density of 7×10^3 cells per well and cultured with α-MEM (Gibco Laboratories, Grand Island, NY) containing 10% FBS (HyClone Laboratories, Logan, UT) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). To determine the role of rhBMP-2 in the myogenic differentiation, we also used a clonal rat cell line of skeletal muscle myoblasts (L6 cells) (43) obtained from the Japanese Cancer Research Resources Bank (Tsukuba Science City, Japan). L6 cells were maintained in DMEM (Gibco Laboratories) supplemented with 10% FBS and antibiotics. L6 cells were plated in 48-well tissue culture plates (Costar Corp., Cambridge, MA) at a cell density of 1×10^4 cells per well. In some experiments, L6 cells were cultured with DMEM containing 2% FBS to induce differentiation of myoblasts (33).

Growth Experiments

After C26 and C20 cells were cultured for 3 d with various concentrations of rhBMP-2, the cells were detached from the culture dishes by incubating with a trypsin/EDTA solution (0.05% trypsin; 0.02% EDTA), and the cell number was counted using a hemocytometer.

Alkaline Phosphatase Activity

Alkaline phosphatase (ALP) activity was determined by an established technique using *p*-nitrophenyl-phosphate as a substrate (26). Protein concentration was determined using a BCA protein assay reagent (Pierce Chemical Co., Rockford, IL).

3'5'-cAMP Production in Response to Parathyroid Hormone

To determine 3',5'-cAMP production in response to parathyroid hormone (PTH), cells were preincubated for 20 min with α-MEM containing 0.5% BSA and 1 mM 3-isobutyl-1-methylxanthine. After preincubation media were removed, cells were incubated for 8 min with 200 ng/ml of human PTH (hPTH(1-34)) (provided by Dr. Hori, Toyo Jozo Co., Shizuoka, Japan) dissolved in the same culture media. The cAMP concentration in the cell layers was determined by RIA using a cAMP assay kit (Yamasa Co., Chiba, Japan).

Myogenic Differentiation

Myotubes appearing in the C26 cells were detected by immunoreactivity to desmin, a muscle-specific intermediate filament. To detect desmin, cells were fixed for 10 min with a cold acetone/ethanol mixture (50:50), and stained for desmin by an indirect immunoperoxidase technique using a mouse anti-cow desmin mAb (Labsystems, Helsinki, Finland). The desmin-positive cells were visualized using a biotinylated anti-mouse IgG antibody (Vector Laboratories, Burlingame, CA). Myotubes appearing in the L6 cells were identified by morphology according to the method described previously (43).

Northern Blot Hybridization of ALP and Collagens

Total RNA was isolated by the guanidine thiocyanate-cesium chloride method (16). 20 μg of the isolated RNA were electrophoresed in a 1.2% agarose-formaldehyde gel, and blotted onto Hybond-N membranes (Amersham International, Amersham, UK). The membranes were hybridized with ^{32}P-labeled cDNA probes at 65°C in a rapid hybridization buffer (Amersham International) and washed in 0.1× SSC buffer containing 0.1% SDS at the same temperature. cDNA probes used were those for rat ALP (23), rat procollagen α1(I) (6), and α1(III) (12), and human β-tubulin.

Measurement of Osteocalcin Synthesis

The C26 cells were treated for 3, 6, or 9 d with or without 1 μg/ml of rhBMP-2. The culture was incubated for the last 24 h of each culture period with or without 2×10^{-8} M 1α,25$(OH)_2D_3$. Northern blot analysis of osteocalcin was performed as described above using a rat osteocalcin cDNA probe (3). The amount of osteocalcin secreted into the culture media was determined by RIA using a rat osteocalcin assay kit (Biomedical Technologies Inc., Stoughton, MA).

Results

Cell Proliferation

Fig. 1 shows the effect of rhBMP-2 on the growth of C26 and C20 cells. The cells were cultured with graded concentrations of rhBMP-2, then counted on day 3. At dose levels >10 ng/ml, rhBMP-2 increased the growth of the C26 cells, but it inhibited the proliferation of the C20 cells dose dependently (Fig. 1).

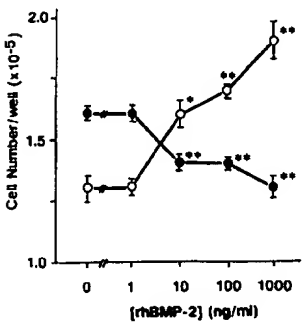

Figure 1. Dose-response effects of rhBMP-2 on the growth of C26 (○) and C20 (●) cells. Cells were plated in 24-well plates and cultured for 3 d with graded concentrations of rhBMP-2. The cells were counted as described in Materials and Methods. Data are means ± SD of three wells. Significantly different from the control without rhBMP-2. (*$p < 0.05$; **$p < 0.01$.)

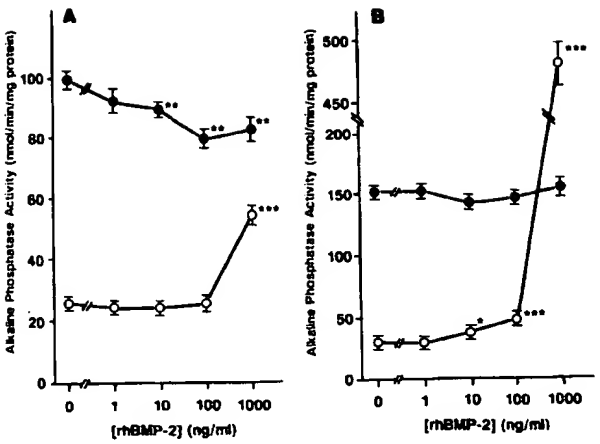

Figure 2. Dose-response effects of rhBMP-2 on ALP activity in C26 (○) and C20 (●) cells. Cells were cultured with graded concentrations of rhBMP-2 for 3 (*A*) and 6 d (*B*). ALP activity was determined by the method described in Materials and Methods. Data are means ± SD of three wells. Significantly different from the control without rhBMP-2. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.)

ALP Activity

C20 cells exhibited basal activity of ALP approximately five times higher than C26 cells in the absence of rhBMP-2. rhBMP-2 stimulated ALP activity in C26 cells but not in C20 cells. After C26 cells were treated for 3 d with 1 μg/ml of rhBMP-2, ALP activity was increased to approximately twice the basal level (Fig. 2 *A*). On day 6, rhBMP-2 increased ALP activity at concentrations <1 μg/ml. The enzyme activity attained a level 16 times higher than the control level when 1 μg/ml of the peptide was added (Fig. 2 *B*). In C20 cells, in contrast, treatment with >10 ng/ml of rhBMP-2 slightly decreased the ALP activity on day 3 (Fig. 2 *A*). Treatment of C20 cells with rhBMP-2 for 6 d did not induce any significant changes in the enzyme activity at any concentrations tested (Fig. 2 *B*).

L6 cells showed a low but detectable ALP activity after they were cultured for 7 d (24.0 ± 1.8 nmol/min per mg protein). Treatment of L6 cells with rhBMP-2 for 6 d increased the enzyme activity to 29.3 ± 0.4 at 10 ng/ml and 32.2 ± 1.9 at 100 ng/ml, but decreased it to 12.5 ± 0.7 nmol/min per mg protein at 1 μg/ml.

PTH-dependent cAMP Production by C26 and C20 Cells

Both C26 and C20 cells produced cAMP in response to 200 ng/ml of hPTH(1-34), but in the absence of rhBMP-2 the fold induction was higher in C20 cells than C26 cells on both days 3 and 6 (Table I). Treatment with rhBMP-2 dose-dependently increased cAMP production in the two osteoblastic cells, but the C26 cells appeared more sensitive to rhBMP-2 than the C20 cells. On day 6, the maximal increase by 1 μg/ml of rhBMP-2 was 151-fold in the C26 cells and 58-fold in C20 cells. In L6 cells, there was no increase in the cAMP production in response to PTH in the presence or absence of rhBMP-2.

mRNA Expression of ALP and Collagens

Northern blot analysis showed that rhBMP-2 increased the steady state level of the expression of ALP mRNA approximately twofold in C26 cells, but did not increase it in C20 cells (Fig. 3). There was no appreciable change in the expression of type I and type III procollagens and β-tubulin mRNAs in the C20 and C26 cells treated with rhBMP-2 (Fig. 3).

Osteocalcin Synthesis

Control C26 cells that were cultured for 3–9 d without rhBMP-2 synthesized neither detectable levels of osteocalcin mRNA nor protein even in the presence of 10^{-8} M $1\alpha,25\text{-}(OH)_2D_3$ (Fig. 4, *A* and *B*). Treatment of C26 cells with 1 μg/ml of rhBMP-2 for 3–9 d greatly increased the expres-

Table I. Effects of rhBMP-2 on the cAMP Production Stimulated by PTH in C20 and C26 Cells

		Amounts of cAMP produced					
		Day 3			Day 6		
Cell line	rhBMP-2	PTH(−)	PTH(+)	PTH(+)/PTH(−) ratio	PTH(−)	PTH(+)	PTH(+)/PTH(−) ratio
	ng/ml				*pmol/well*		
C26	0	1.3 ± 0.0	2.5 ± 0.0	1.9	0.8 ± 0.0	12.2 ± 0.1	15.0
	1	1.6 ± 0.1	3.1 ± 0.2	2.0	0.8 ± 0.0	10.1 ± 0.1	13.0
	10	1.5 ± 0.0	4.2 ± 0.4	2.8	0.8 ± 0.0	14.3 ± 0.1	17.9
	100	1.7 ± 0.0	20.0 ± 1.5	11.5	1.0 ± 0.0	60.4 ± 2.4	62.5
	1,000	2.5 ± 0.1	110.0 ± 7.6	48.2	2.5 ± 0.1	375.0 ± 9.8	151.4
C20	0	1.4 ± 0.2	4.2 ± 0.5	3.0	2.4 ± 0.1	62.4 ± 0.8	27.9
	1	1.1 ± 0.1	4.8 ± 0.4	4.1	2.0 ± 0.1	48.0 ± 2.0	24.7
	10	1.5 ± 0.1	6.3 ± 0.2	4.2	2.5 ± 0.2	66.9 ± 1.3	26.7
	100	1.4 ± 0.1	14.7 ± 1.7	10.7	2.1 ± 0.1	72.7 ± 1.6	35.3
	1,000	1.8 ± 0.3	22.0 ± 1.2	12.5	1.6 ± 0.1	92.5 ± 4.1	58.3

Cells were cultured for 3 or 6 d with graded concentrations of rhBMP-2. On days 3 and 6, cells were treated for 8 min with 200 ng/ml of hPTH(1-34) and the amount of cAMP produced was determined. Data are means ± SEM of three wells.

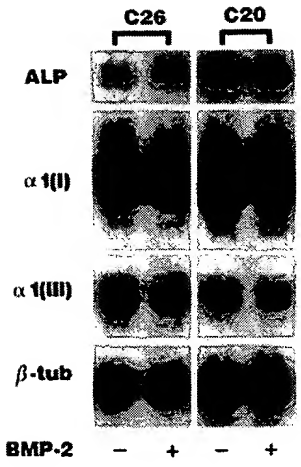

Figure 3. Northern blot analysis of the mRNA expression of ALP and procollagens of α1(I) and α1(III). Total RNAs were isolated from C26 and C20 cells after treatment for 3 d with 1 μg/ml of rhBMP-2. 20 μg of total RNA were electrophoresed in agarose gel and hybridized with the respective ^{32}P-labeled cDNA probes as described in Materials and Methods. *β-tub*, β-tubulin.

sion of osteocalcin mRNA (Fig. 4 *B*). On day 3, a low level of osteocalcin mRNA was expressed only in the 1α,25-$(OH)_2D_3$-treated cells. On days 6–9, the levels of mRNA increased even in the absence of 1α,25$(OH)_2D_3$, and its expression was strikingly increased by the treatment with 1α, 25$(OH)_2D_3$ for 24 h (Fig. 4 *B*). The amounts of osteocalcin secreted into the culture media were closely related to the mRNA levels of osteocalcin (Fig. 4, *A* and *B*).

Myogenic Differentiation

When C26 cells became confluent, some of them spontaneously differentiated into desmin-positive myotubes (Fig. 5 *A*). Adding rhBMP-2 dose-dependently decreased the number of desmin-positive myotubes at dose levels >1 ng/ml of the peptide (Figs. 5 *B* and 6 *A*). Numerous myotubes also appeared when L6 cells were cultured with the inducing culture media (Fig. 5 *C*). Treatment of L6 cells with rhBMP-2 at higher concentrations than 1 ng/ml similarly reduced the number of myotubes dose dependently (Figs. 5 *D* and 6 *B*).

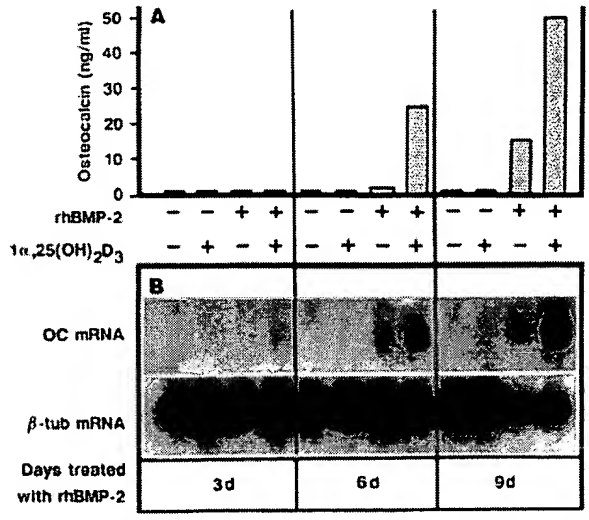

Figure 4. Induction of osteocalcin synthesis by rhBMP-2 in C26 cells. C26 cells (7.9×10^4 cells) were inoculated in 60-mm^2 culture dishes. After preculturing the cells for 24 h, rhBMP-2 (1 μg/ml) was added and the cells were further cultured for 3, 6, or 9 d. The dish was treated for the last 24 h of each culture period with or without 2×10^{-8} M 1α,25$(OH)_2D_3$. (*A*) The amount of osteocalcin secreted into culture media as determined by RIA using the method described in Materials and Methods. (*B*) mRNA expression for osteocalcin. 20 μg of total RNA isolated from each sample were electrophoresed in agarose gel and hybridized with a ^{32}P-labeled cDNA probe for osteocalcin as described in Materials and Methods. *OC*, osteocalcin, *β-tub*, β-tubulin.

Effects of TGF-β1 on Osteoblastic and Myogenic Differentiation

BMP-2 is a member of the TGF-β superfamily (41). We examined the effect of TGF-β1 on osteoblastic and myogenic differentiation to determine whether the effects shown in this study are specific to BMP-2. Like BMP-2, TGF-β1 dose-dependently decreased the number of myotubes appearing in C26 cells (Fig. 7 *C*). However, unlike BMP-2, treatment of C20 cells with 0.1–10 ng/ml of TGF-β1 for 6 d decreased ALP activity dose dependently (Fig. 7 *A*). TGF-β1 also suppressed ALP activity in C26 cells, though the inhibitory effect was smaller than in C20 cells (Fig. 7 *A*). TGF-β1 showed no significant effect on the PTH-dependent cAMP production in C26 cells at any concentrations tested (Fig. 7 *B*), but it increased PTH responsiveness in C20 cells (Fig. 7 *B*). Treatment of C26 cells for 6 d with 0.1–10 ng/ml of TGF-β1 induced no osteocalcin production even in the presence of 1α, 25$(OH)_2D_3$.

Discussion

The present study clearly demonstrates that the recombinant BMP-2 poly-peptide affects proliferation and differentiation of certain osteoblastic cells in vitro. To determine if rhBMP-2 had differential effects on osteoblasts at varying stages of differentiation, we used two clonal osteoblast-like cell lines at different stages of differentiation: C26 cells are osteoprogenitor cells, also retaining potentials to differentiate into muscle cells and adipocytes; C20 cells are more differentiated osteoblast-like cells (44). rhBMP-2 stimulated the growth of C26 cells but slightly inhibited the proliferation of C20 cells. The stimulatory effect of rhBMP-2 on the osteoblastic phenotype was much greater in C26 cells than in C20 cells. This indicates that rhBMP-2 preferentially stimulates proliferation and differentiation of osteoprogenitor cells.

At present, osteocalcin is the only known bone-specific protein produced by osteoblasts (11). This protein is reported to appear in a later stage of osteoblast differentiation (2, 34, 45), presumably in mature osteoblasts, whereas ALP (2, 34, 45), PTH receptors (29), and type I collagen (31, 34, 45) appear even in less differentiated osteoblasts. Although C26 cells have already acquired a low level of the latter characteristics, but they lack osteocalcin synthesis in an unstimulated state. It is significant that rhBMP-2 induced osteocalcin mRNA expression and its protein synthesis in C26 cells. Apparently rhBMP-2 induced differentiation of immature osteoblastic cells into mature osteoblasts not only by increasing the expression of the constitutive phenotype present in progenitor cells, but also by inducing the expression of novel genes present only in mature osteoblasts. rhBMP-2 is the first cytokine that is capable of inducing osteocalcin mRNA expression and its protein synthesis. Whether other bone

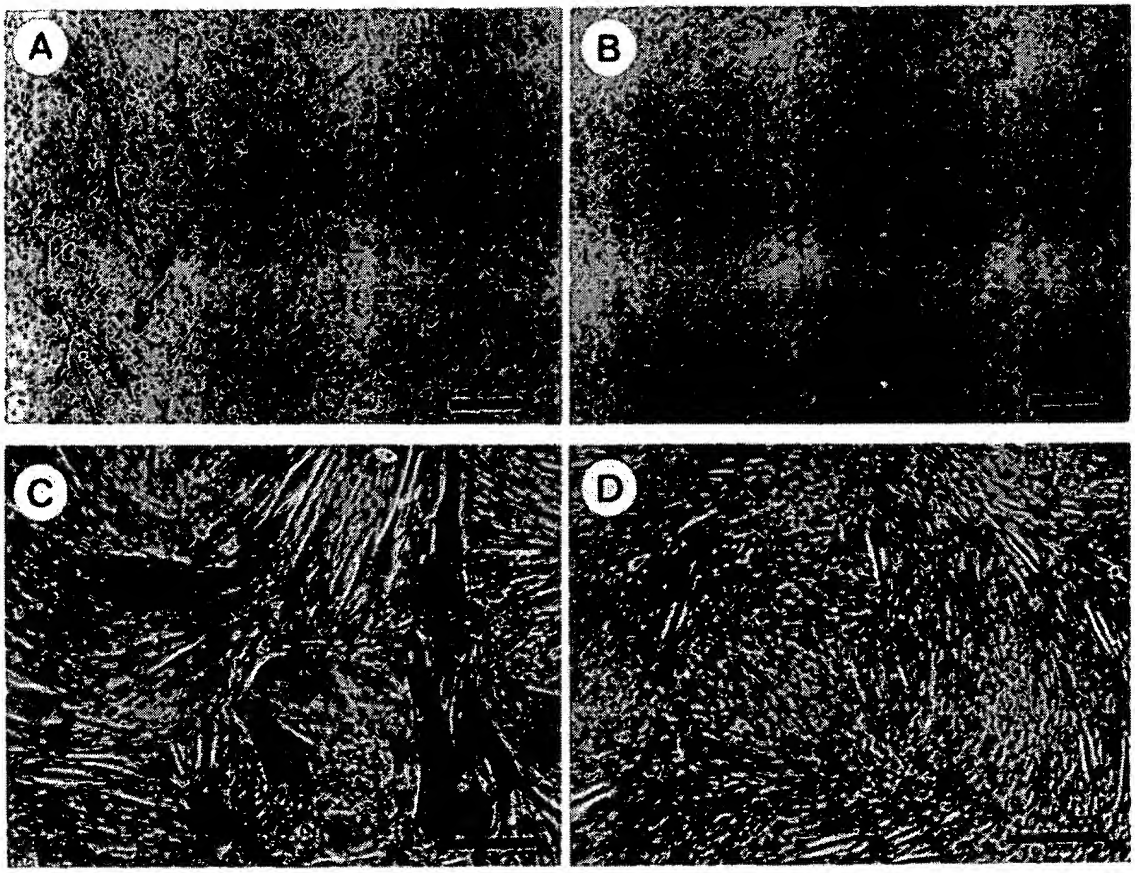

Figure 5. Effects of rhBMP-2 on the development of myotubes in C26 and L6 cells. (*A* and *B*) C26 cells were immunostained with an anti-desmin antibody as described in Materials and Methods. Numerous desmin-positive elongated myotubes are seen in the C26 cells cultured for 7 d in the absence of rhBMP-2 (*A*). Note that there is a marked decrease in the number of desmin-positive myotubes in the C26 cells cultured with rhBMP-2 (100 ng/ml) for 6 d (*B*). (*C* and *D*) Phase-contrast features of L6 cells. The cells were cultured with the media containing 10% FBS for the first 3 d then with the media containing 2% FBS for the last 3 d. Numerous multinucleated myotubes are seen in the L6 cells cultured in the absence of rhBMP-2 (*C*). Treatment with rhBMP-2 (100 ng/ml) for the last 3 d greatly inhibited the appearance of myotubes (*D*). Bars, 200 μm.

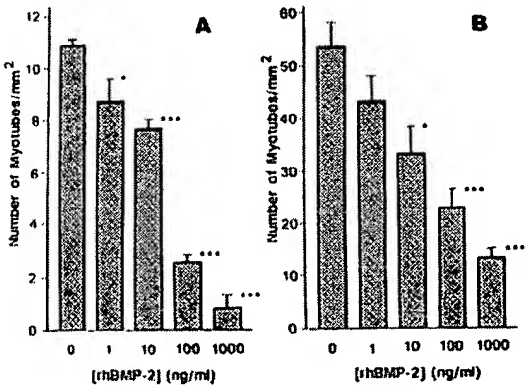

Figure 6. Dose-response effects of rhBMP-2 on the development of myotubes in C26 cells (*A*) and L6 cells (*B*). Myotubes appearing in each cell line were identified as described in Materials and Methods. The number of myotubes was counted in 6.25-mm² at the central region of each well. Data are means ± SD of three wells. Significantly different from the control without rhBMP-2. (*$p < 0.05$; ***$p < 0.001$.)

growth factors have similar effects in inducing osteocalcin synthesis remains to be elucidated in the future.

Cells of the osteoblastic phenotype make a significant quality of type I collagen. In the present study, treatment with rhBMP-2 induced no appreciable change of expression of type I and type III procollagen mRNAs in C26 and C20 cells. Collagen synthesis measured by [³H]proline incorporation was not significantly stimulated by rhBMP-2 (100 ng/ml) in C26 cells and in calvarial cultures (unpublished data). No stimulation occurred in the mRNA expression for type I procollagen in the mouse clonal embryonic fibroblastic cells (C3H10T1/2) (unpublished data), which responded to rhBMP-2 in increasing their ALP activity and PTH responsiveness (9). It thus appears that BMP-2 has no appreciable effect on collagen synthesis, at least in the cell lines we used.

The minimal doses of rhBMP-2 that affected proliferation and differentiation of C26 cells were much higher than those of other cytokines and growth factors. Also, the effective doses of rhBMP-2 necessary to induce ALP activity were >10 ng/ml in C3H10T1/2 cells (9) and mouse osteoblast-like

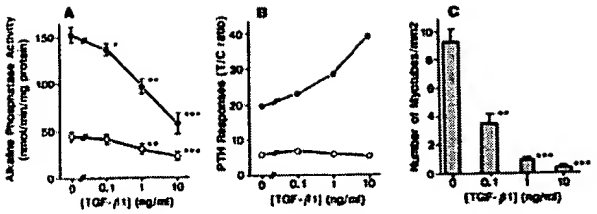

Figure 7. Effects of TGF-β1 on osteoblastic and myogenic differentiation. (*A*) Dose-response effects of TGF-β1 on ALP activity in C26 (○) and C20 (●) cells. After the cells were cultured with graded concentrations of TGF-β1 for 6 d, the ALP activity was determined by the method described in Materials and Methods. Data are means ± SD of three wells. (*B*) Dose-response effects of TGF-β1 on the cAMP production stimulated by PTH in C26 (○) and C20 (●) cells. The cells were cultured for 6 d with graded concentrations of TGF-β1. Then the amount of cAMP produced was determined as described in Materials and Methods, after the cells were treated for 8 min with or without 200 ng/ml of hPTH(1-34). The PTH response was expressed as the ratio of cAMP production by PTH-stimulated cells to that by unstimulated cells. Data are means of three wells in each group. (*C*) Dose-response effects of TGF-β1 on myogenic differentiation in C26 cells. Myotubes were identified as described in Materials and Methods. The number of desmin-positive cells was counted in 6.25-mm² at the central region of each well. Data are means ± SD of three wells. Significantly different from the control without TGF-β1. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.)

MC3T3-E1 cells (10) (unpublished data). However, this low responsiveness might not be due to the low specific activity of BMP-2. This may be explained by the difference between the native BMP-2 and the recombinant one, since it requires more rhBMP-2 than the native BMPs to induce similar ectopic bone formation (40). Alternatively, it may be due to the sticky nature of BMP-2. Further comparative studies using native and recombinant BMPs are needed to clarify this point.

During the process of ectopic bone formation after bone-inducing factors are implanted into muscular tissues, these factors appear to alter the differentiation of muscle cells around the sites applied. The present study clearly demonstrates that rhBMP-2 inhibits myogenic differentiation in vitro. Apparently BMP-2 has opposite actions on osteogenic and myogenic differentiation in pluripotent cells: rhBMP-2 stimulates differentiation of mesenchymal cells into osteoblastic cells and inhibits differentiation of those cells into muscle cells. It is reported that cartilage formation precedes the osteoblast differentiation in the process of ectopic bone formation induced by BMP-2 (27, 28). Since myogenic cells are induced to differentiate into chondrogenic cells in response to bone matrix in vitro (18, 19, 24, 32), it will be interesting to determine whether rhBMP-2 induces differentiation of C26 cells and L6 cells into chondrogenic cells. This possibility is currently under investigation.

BMP-2 is a member of the TGF-β superfamily (41). To determine whether the effects shown in this study are specific to BMPs, we compared the effects of rhBMP-2 and TGF-β1 on osteogenic and myogenic differentiation in C26 and C20 cells. Like BMP-2, TGF-β1 suppressed myogenic differentiation in C26 cells. However, unlike BMP-2, TGF-β1 decreased ALP activity in both C26 and C20 cells. TGF-β1 stimulated PTH responsiveness in C20 cells, but it induced neither PTH responsiveness nor osteocalcin production in C26 cells. In vivo studies (8, 22) have indicated that TGF-β injected into periosteal regions induced new bone formation in rats, but several in vitro experiments have revealed that the effects of TGF-β on ALP activity and PTH responsiveness varied among the cells tested (4, 7, 20, 21). The inconsistency of the in vitro data suggests that the action of TGF-β on osteoblast differentiation depends on the stage of differentiation of the osteoblast-like cells used and the culture conditions. The inhibitory effects of TGF-β on myogenic differentiation have also been demonstrated in other myogenic cells (5, 17). Together, these results indicate that both BMP-2 and TGF-β similarly inhibit myogenic differentiation, but they have different actions in osteoblast differentiation.

Although the precise role of the respective BMPs and their mutual interaction in osteoblast differentiation have to be elucidated in the future, it is interesting that in the recent finding reported by Lyons et al. (15), BMP-2, Vgr-1 (BMP-6), TGF-β1 and TGF-β2 mRNAs are expressed in different populations of mesenchymal cells in the developing skeletal system. They also proposed the importance of the coordinated expression of several members of the TGF-β superfamily for the control of progression of specific cell types through the differentiation pathways (15). It is important to determine the specific actin and the mutual interaction of various BMPs and TGF-βs to understand the precise regulatory mechanisms of osteoblast differentiation.

In conclusion, rhBMP-2 is a bone induction factor that induces differentiation of osteoblast progenitor cells into mature osteoblast with the ability to synthesize osteocalcin. Also, rhBMP-2 inhibits myogenic differentiation. So it appears that BMP-2 is involved in both in vivo osteoblastic and myogenic differentiation at the site of implantation. Whether BMP-2 affects other cell differentiation events including chondrogenesis and adipogenesis is of considerable interest and is under investigation in our laboratories.

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Recombinant Human Bone Morphogenetic Protein-2 Induces Osteoblastic Differentiation in W-20–17 Stromal Cells

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ABSTRACT. To better understand the *in vivo* bone-inductive properties of recombinant human (rh) BMP-2, we examined the ability of the protein to alter the phenotype of a bone marrow stromal cell line, W-20–17. rhBMP-2 increased alkaline phosphatase activity in W-20–17 cells in a dose-responsive manner in the absence of an effect on proliferation. The induction of alkaline phosphatase activity was not apparent until 12 h after rhBMP-2 treatment had begun and was effectively eliminated by cotreatment with cycloheximide, suggesting a requirement for protein synthesis. Continued treatment of W-20–17 cells with rhBMP-2 for 8 days resulted in a significant increase, compared to control cultures, in the production of cellular cAMP in response to a PTH challenge. In addition, 4-day treatment with rhBMP-2 induced osteocalcin levels in W-20–17 cells. These results indicate that rhBMP-2 induces the expression of several markers associated with the osteoblast phenotype in W-20–17 cells and raises the possibility that BMP-2 may be involved in the differentiation of osteoblasts from progenitor cells resident in bone marrow. (*Endocrinology* **130**: 1318–1324, 1992)

BONE formation, as it occurs in fracture healing and embryonic skeletal development, is thought to begin with the recruitment of mesenchymal stem cells, which differentiate to osteoblasts under the influence of one or more osteogenic factors. While the precise lineage leading to the osteoblast phenotype is not clearly established, there is substantial evidence to indicate that osteoblast precursor cells are present in bone periosteum and bone marrow stroma. For example, clonal periosteal cells have been isolated that will differentiate into osteoblastic cells in culture (1, 2). In addition, injection of transforming growth factor-β (TGFβ) subperiosteally in rats will induce osteogenesis at the injection site (3, 4). Furthermore, bone marrow stromal cells have been shown to acquire the osteoblast phenotype in culture (5, 6) and form bone *in vivo* after implantation in diffusion chambers (7, 8). It is not known whether osteoblast progenitors from the periosteum and bone marrow are derived from a common stem cell or exist as separate lineages. The intimate association in marrow of hematopoietic and stromal cells raises the possibility that their interactions may regulate not only hematopoiesis, but also stromal cell differentiation, including osteogenesis.

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The proteins contained in demineralized bone matrix have long been recognized as osteoinductive agents *in vivo* (9). Demineralized bone matrix, when implanted ectopically in rats, induces the formation of new bone through a developmental mechanism analogous to that observed during embryonic bone formation or fracture repair in adult animals. The ectopically induced bone includes a periosteum and bone marrow stroma, suggesting that the osteoprogenitors populating these areas are responsive to bone-derived osteogenic factors. We have recently purified and molecularly cloned the genes for several proteins with osteogenic potential (10, 11). One of these recombinant proteins, BMP-2, is sufficient by itself to induce ectopic bone formation *in vivo* (10, 12). In this study we have examined the effects of purified recombinant human (rh) BMP-2 on the phenotype of a bone marrow stromal cell line, W-20–17. We report here a detailed analysis of the effects of BMP-2 on W-20–17 cells and show that BMP-2 induces alkaline phosphatase activity, osteocalcin synthesis, and PTH sensitivity in this cell line.

Materials and Methods

Differentiation factors

Bioactive rhBMP-2 was produced by Chinese hamster ovary (CHO) cells and purified as described previously (12). The

recombinant BMP-2 preparations used in this study were estimated to be greater than 90% pure, based on silver-stained gels. Conditioned media containing rhBMP-4 were produced by CHO cells. 1,25-Dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] was generously provided by Dr. M. R. Uskokovic (Hoffman La-Roche, Nutley, NJ). Dexamethasone, hydrocortisone, and PTH, fragment 1–34 (PTH) were purchased from Sigma (St. Louis, MO).

W-20 cell culture

An oligoclonal W-20 cell pool derived from the bone marrow of a w^{++} mouse strain was obtained from Drs. S. Tsai and D. G. Nathan (Harvard Medical School, Boston, MA). These cells were cloned by limiting dilution to obtain clonal W-20 cell lines. W-20 clone 17 cells (W-20–17) were used in this study. Cells were cultured in Dulbecco's Modified Eagle's Medium (DME; Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS; Hazleton, Denver, PA).

Alkaline phosphatase assay

W-20–17 cells were plated in 96-well dishes at 10^4 cells/well. Twenty-four hours after plating, the medium was replaced with fresh medium alone or medium containing the agent to be tested. After an additional 24-h incubation, cells were washed and then lysed by freeze-thawing in water. Cell lysates were assayed for alkaline phosphatase activity in 50 mM glycine, 0.05% Triton X-100, 4 mM $MgCl_2$, and 5 mM *p*-nitrophenol phosphate, pH 10.3, for 30 min at 37 C. The spectrophotometric absorbance at 405 nm was compared to that of *p*-nitrophenol standards to estimate alkaline phosphatase activity in the samples. Activities were normalized to cell protein, as measured by the bicinchoninic acid assay (Pierce, Rockford, IL), using immunoglobulin G as a standard. Under these conditions, the alkaline phosphatase assay reaction was linear.

[^{3}H]Thymidine incorporation

W-20–17 cells were grown to confluence with DME-10% FCS in 96-well dishes and serum starved for 24 h with DME-0.5% FCS. Cells were then incubated with 10% serum, 0.5% serum, or rhBMP-2 and 0.5% serum for 24 h. [^{3}H]Thymidine was included in the last 4 h of this incubation. The cells were washed, and the incorporated [^{3}H]thymidine was measured by liquid scintillation counting.

[^{35}S]Methionine incorporation

To determine the extent of protein synthesis inhibition by cycloheximide, W-20–17 cells at about 80% confluence were pretreated with different doses of cycloheximide for 3 h, rinsed, and then preincubated for 10 min in (−)methionine medium. After this preincubation, the cells were pulsed for 15 min with [^{35}S]methionine in (−)methionine medium and then chased for 24 h with DME-10% FCS containing different doses of cycloheximide. [^{35}S]Methionine incorporation into protein was measured by liquid scintillation counting of the trichloroacetic acid-precipitable material from these cells.

Tests for calcification of W-20–17 cells

To test for *in vitro* calcification, confluent W-20–17 cells were cultured in DME-10% FCS with or without 100 ng/ml rhBMP-2 and supplemented with 50 μg/ml ascorbic acid and 7 mM β-glycerophosphate for up to 10 days. The cells were fixed and stained for calcification with the Von Kossa procedure. To assay *in vivo* calcification, trypsinized W-20–17 cells were washed free of serum and injected into 1-cm diffusion chambers (10^7 cells/chamber) equipped with 5-μm pore filters (Millipore, Bedford, MA). The diffusion chambers were implanted sc or ip in rats for 5 or 4 weeks, respectively. Excised chambers were fixed, embedded, sectioned, stained, and examined histologically for calcification.

Northern blot analysis

Cytoplasmic RNA was isolated from W-20–17 cells and ROS cells by the Nonidet P-40 lysis method (11). Poly(A)-containing RNA was selected by oligo(dT)-cellulose chromatography. The mRNA was electrophoresed on a 1% formaldehyde agarose gel, blotted, and hybridized with ^{32}P-labeled cDNA probe under stringent conditions.

cAMP production in response to PTH

Confluent W-20–17 cells were preincubated with 1 mM 3-isobutyl-1-methylxanthine for 20 min, followed by a 10-min incubation with 400 ng/ml PTH, fragment 1–34 (PTH). The cAMP concentration in cells extracted with 10% trichloroacetic acid was determined by RIA, using a cAMP assay kit (DuPont, Wilmington, DE).

Osteocalcin production

Confluent W-20–17 cells were treated with or without rhBMP-2 for 4 days. On the fourth day of treatment, 24-h conditioned media were collected and assayed for osteocalcin by RIA, using a mouse osteocalcin assay kit (Biomedical Technologies, Inc., Stoughton, MA).

Statistical analysis

Results are expressed as the mean ± SEM. The two-tailed Student's *t* test was used to determine statistical significance of differences between means unless otherwise indicated.

Results

To understand the bone-inductive properties of rhBMP-2 *in vivo*, several potential osteoblast progenitor cell types were screened for *in vitro* rhBMP-2-induced activities characteristic of the osteoblast phenotype. This search revealed that oligoclonal W-20 cells derived from mouse bone marrow stroma exhibited increased alkaline phosphatase activity in response to rhBMP-2 treatment. Since this induction appeared heterogeneous in this oligoclonal cell line, clones of W-20 were prepared and assayed for rhBMP-2-induced alkaline phosphatase activity. By this procedure, W-20 clone 17 cells (W-20–17), shown in Fig. 1, were selected for subsequent experiments, and all data reported herein use this W-20 clone. As shown in Fig. 2, W-20–17 cells exhibited low basal levels of alkaline phosphatase activity in culture. When subconfluent W-20–17 cells were treated with purified

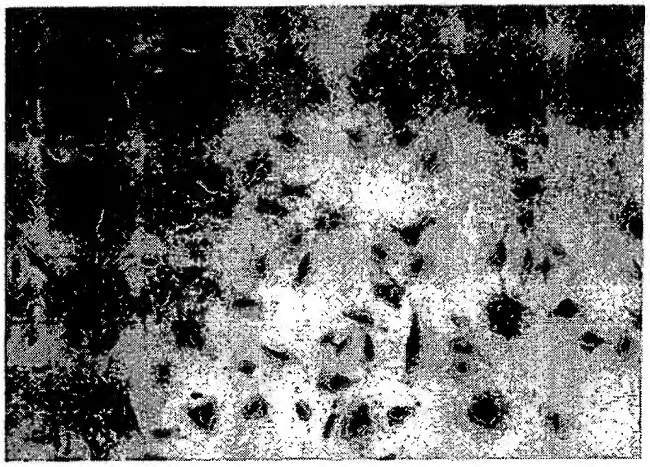

FIG. 1. W-20-17 cells cultured in DME-10% FCS, fixed in 4% formaldehyde, and stained with toluidine blue (×200).

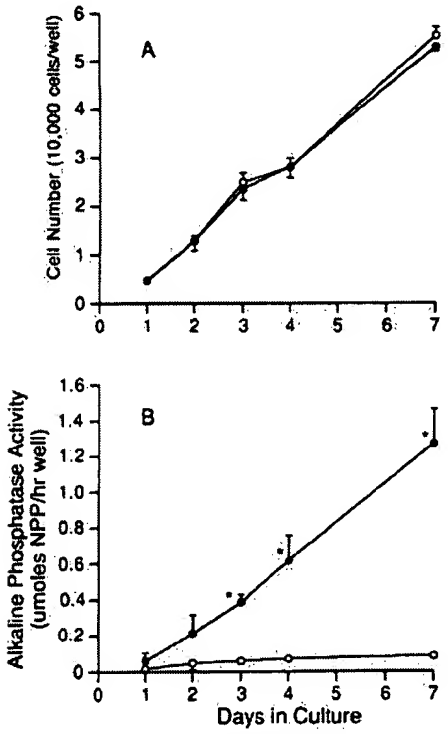

FIG. 2. Effect of rhBMP-2 on cell number and alkaline phosphatase activity of W-20-17 cells. Cultures were plated at 2000 cells/well in 96-well plates and grown for 7 days with (●) or without (○) 100 ng/ml rhBMP-2. After 1, 2, 3, 4, and 7 days in culture, cells were trypsinized and counted with a hemacytometer (A) or assayed for alkaline phosphatase activity (B) in triplicate. Results are expressed as the mean ± SEM of three experiments. ★, $P < 0.01$.

rhBMP-2, alkaline phosphatase activity was increased above basal levels (Fig. 2). However, cell number was not significantly affected by treatment (Fig. 2). To confirm the absence of a growth stimulatory effect, rhBMP-2 was tested for its ability to stimulate [^{3}H]thymidine incorporation in W-20-17 cells. As shown in Fig. 3, when

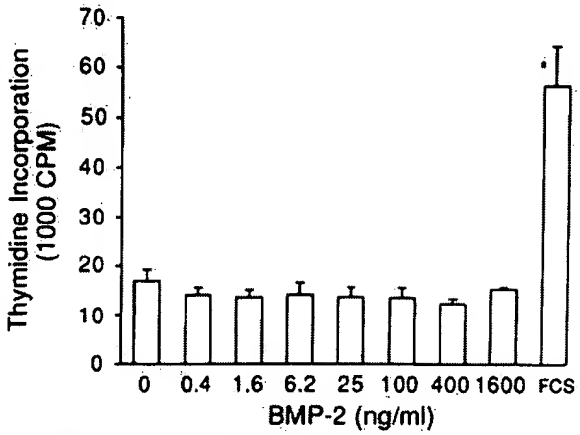

FIG. 3. Effect of rhBMP-2 or serum on [^{3}H]thymidine incorporation in W-20-17 cells. Confluent W-20-17 cells were cultured in 0.5% serum for 24 h and then incubated with 10% serum or rhBMP-2 and 0.5% serum for an additional 24 h in quadruplicate. [^{3}H]Thymidine was included in the last 4 h of this incubation. Results are expressed as the mean ± SEM of three experiments. ★, $P < 0.01$.

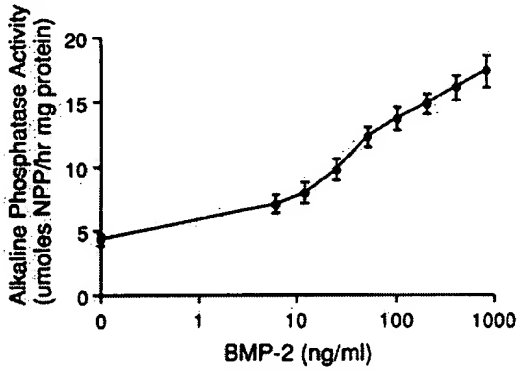

FIG. 4. Dose response of rhBMP-2-induced alkaline phosphatase activity in W-20-17 cells. Confluent W-20-17 cells treated with different concentrations of rhBMP-2 for 24 h were assayed for alkaline phosphatase activity in triplicate using *p*-nitrophenol phosphate as a substrate. Results are expressed as the mean ± SEM of seven experiments. All doses shown resulted in statistically significant increases in alkaline phosphatase activity above the control value ($P < 0.01$). NPP, *p*-Nitrophenol phosphate cleaved.

serum-starved W-20-17 cells were treated with medium containing 10% serum for 24 h, [^{3}H]thymidine incorporation was increased 3-fold above basal levels. In contrast, between 0.4–1600 ng/ml rhBMP-2 caused no change in [^{3}H]thymidine incorporation. These findings confirmed that rhBMP-2 was not exerting a mitogenic effect in W-20-17 cells.

To test the dose responsiveness of rhBMP-2-stimulated alkaline phosphatase activity, confluent W-20-17 cells were treated with varied concentrations of rhBMP-2 for 24 h before assay. As shown in Fig. 4, rhBMP-2 stimulated alkaline phosphatase activity in a dose-responsive manner, beginning at 6 ng/ml. However, a maximal response was not attained with concentrations of rhBMP-2 as great as 800 ng/ml, and therefore, it was

not possible to estimate an ED_{50} value. The observation that relatively high concentrations of rhBMP-2 were unable to achieve a maximal effect raised the possibility that rhBMP-2 might be labile under these conditions, thus artifactually lowering the delivered dose. To address this possibility, media containing rhBMP-2 (0–1600 ng/ml) were incubated with confluent W-20–17 cells for 24 h before being harvested, diluted 1:1 with fresh medium, and incubated with a second group of W-20–17 cells. The effects of these media on the induction of alkaline phosphatase activity were compared with those of 1:1 diluted control W-20–17-conditioned media (0 ng/ml rhBMP-2) to which rhBMP-2 was added immediately before incubation with the second group of W-20–17 cells. As shown in Fig. 5, while there was some loss of response at low doses of rhBMP-2, greater than 90% of the activity remained intact at doses at or above 100 ng/ml after 24 h in culture with W-20–17 cells. Therefore, the apparent doses of rhBMP-2 required to achieve maximal induction of alkaline phosphatase activity in W-20–17 cells are not artifactually elevated by degradation or inactivation of rhBMP-2.

The time course for rhBMP-2 stimulation of alkaline phosphatase activity in confluent W-20–17 cells is shown in Fig. 6. Significant stimulation was not observed until 12 h after the initiation of rhBMP-2 treatment, suggesting an increase in the expression of alkaline phosphatase protein, rather than an activation of enzymatic activity. To investigate this possibility, we examined the effects of cycloheximide, an inhibitor of protein synthesis, on rhBMP-2-stimulated alkaline phosphatase activity. A 24-h treatment with 2 μg/ml cycloheximide effectively blocked the stimulation of alkaline phosphatase by 100 ng/ml rhBMP-2, decreasing the activity from 13.16 ± 2.09 to 2.38 ± 0.42 μmol *p*-nitrophenol phosphate/h·mg protein (n = 4; $P < 0.001$) without significantly altering

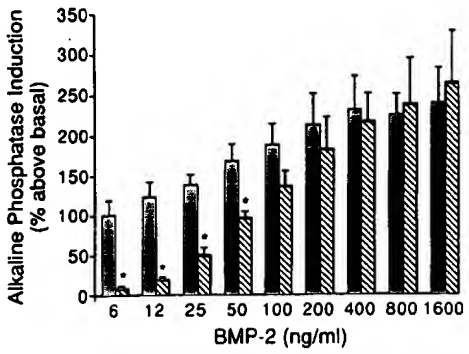

FIG. 5. Activity of rhBMP-2 after W-20–17 cell assay compared to that of fresh rhBMP-2. Confluent W-20–17 cells were treated with fresh rhBMP-2 in conditioned media from untreated W-20–17 cells (□) or with conditioned media from W-20–17 cells treated with rhBMP-2 for 24 h (▨). Alkaline phosphatase activity was assayed in triplicate, using *p*-nitrophenol phosphate as a substrate. Results are expressed as the mean ± SEM of four experiments. ☆, $P < 0.05$.

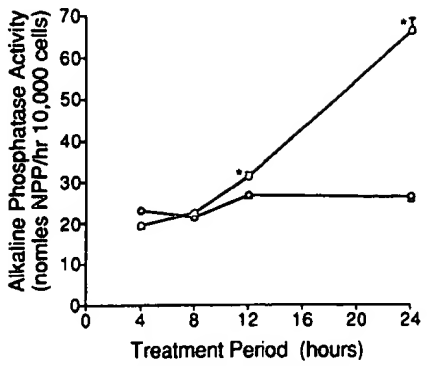

FIG. 6. Time course of rhBMP-2-induced alkaline phosphatase activity in W-20–17 cells. Confluent W-20–17 cells were treated with (○) or without (○) 100 ng/ml rhBMP-2 and assayed at 4, 8, 12, and 24 h for alkaline phosphatase activity in triplicate. Results are expressed as the mean ± SEM of three experiments. ☆, $P < 0.05$. NPP, *p*-Nitrophenol phosphate cleaved.

basal alkaline phosphatase activity (2.41 ± 0.92 *vs.* 4.00 ± 0.92 μmol *p*-nitrophenol phosphate/h·mg protein, with or without cycloheximide, respectively; n = 4; $P > 0.05$). Control experiments confirmed that under these conditions 2 μg/ml cycloheximide inhibited [^{35}S]methionine incorporation by 96%. These results suggest that protein synthesis is required for the stimulation of alkaline phosphatase in W-20–17 cells and argue against a direct activation of the enzyme by rhBMP-2.

The derivation of this cell line from bone marrow and the induction of alkaline phosphatase activity *in vitro* suggested that W-20–17 cells might represent an osteoblast progenitor cell. Therefore, it was of interest to examine W-20–17 cells for other characteristics of the osteoblast phenotype.

W-20–17 cells were tested for the ability to mineralize both *in vitro* and *in vivo*. Confluent W-20–17 cells cultured for 10 days in the presence of 100 ng/ml rhBMP-2, 50 μg/ml ascorbic acid, and 7 mM β-glycerophosphate failed to mineralize, as indicated by Von Kossa staining of fixed monolayers (data not shown). Confluent ROS 17/2.8 cells, a rat osteosarcoma-derived cell line, treated with 50 μg/ml ascorbic acid and 7 mM β-glycerophosphate served as a positive control (data not shown). *In vivo* mineralization was evaluated by implanting diffusion chambers containing 10^7 W-20–17 cells/chamber sc or ip in rats for 35 or 28 days, respectively (13). After the implantation periods, no histological indication of bone formation in the chambers was observed (data not shown). Intraperitoneal implants of primary mouse limb-bud cells served as the positive control in these experiments (data not shown).

Confluent W-20–17 cells treated with rhBMP-2 exhibited a stimulation of adenylate cyclase in response to PTH, which is characteristic of osteoblastic cells. As shown in Table 1, untreated W-20–17 cells showed little

TABLE 1. PTH sensitivity of W-20-17 cells, as indicated by cAMP production

rhBMP-2 pretreatment (ng/ml)	cAMP production (pmol/well)		
	−PTH	+PTH	Δ
0	4.7 ± 0.5	7.8 ± 0.4	3.1 ± 0.6
500	4.1 ± 0.6	141.0 ± 23.0	136.5 ± 22.8

Confluent W-20-17 cells were pretreated for 8 days with or without 500 ng/ml rhBMP-2. On day 8, cells were pretreated for 20 min with 1 mM IBMX, followed by a 10-min treatment with 400 ng/ml PTH. Cellular cAMP was extracted and assayed by RIA. Data are expressed as the mean ± SEM of three experiments. Stimulation of cAMP production by PTH is statistically significant, with or without rhBMP-2 pretreatment ($P < 0.05$, by Student's paired t test). cAMP production in PTH-treated cells is greater with rhBMP-2 pretreatment ($P < 0.05$, by Student's t test with Cochran's adjustment for unequal variances). Δ, Change.

responsiveness to PTH, as indicated by changes in cellular cAMP levels. In contrast, confluent W-20–17 cells treated with 500 ng/ml rhBMP-2 for 8 days exhibited increased PTH sensitivity, which closely resembles the response of osteoblastic cell lines (14, 15).

Furthermore, W-20–17 cells treated with rhBMP-2 tested positively for expression of the bone-specific protein osteocalcin. Whereas confluent W-20–17 cells cultured in the absence of rhBMP-2 for 4 days failed to synthesize levels of osteocalcin mRNA detectable by Northern blot analysis, doses of rhBMP-2 between 5–500 ng/ml induced osteocalcin mRNA (Fig. 7). By comparison, proteoglycan core protein mRNA, a marker of chondroblastic cells, was undetectable under all conditions in the same Northern blots (data not shown). Reprobing the Northern blots for type I collagen mRNA revealed low constitutive levels in both control and rhBMP-2-treated W-20–17 cells (data not shown). Consistent with the observed induction of osteocalcin mRNA, secreted osteocalcin protein was observed in W-20–17 cells treated with 31–1000 ng/ml rhBMP-2 for 4 days (Fig. 7).

Attempts to identify other regulators of alkaline phosphatase activity in W-20–17 cells are summarized in Table 2. Dexamethasone, hydrocortisone, PTH, and 1,25-$(OH)_2D_3$ did not significantly alter the activity of alkaline phosphatase in W-20–17 cells. TGFβ, which has been shown to stimulate or inhibit alkaline phosphatase activity in osteoblastic cells (16, 17) and shares 38% sequence homology with BMP-2, failed to stimulate alkaline phosphatase activity in W-20–17 cells over a broad range of concentrations. There was some indication of a statistically significant inhibitory effect of TGFβ on basal alkaline phosphatase activity, although this did not appear to be dose responsive in the concentration range tested. W-20–17 cells treated simultaneously with TGFβ and rhBMP-2 exhibited slightly less alkaline phosphatase activity than cells treated with rhBMP-2 alone ($P < 0.002$, by Student's paired t test). Retinoic acid had no effect on W-20–17 cell alkaline phosphatase activity. rhBMP-4 was also tested for an effect on W-20–17 cells. In this experiment unpurified conditioned media from CHO cells expressing rhBMP-4 were used. Interestingly, rhBMP-4, with 92% sequence homology to rhBMP-2 (10), was able to stimulate alkaline phosphatase activity.

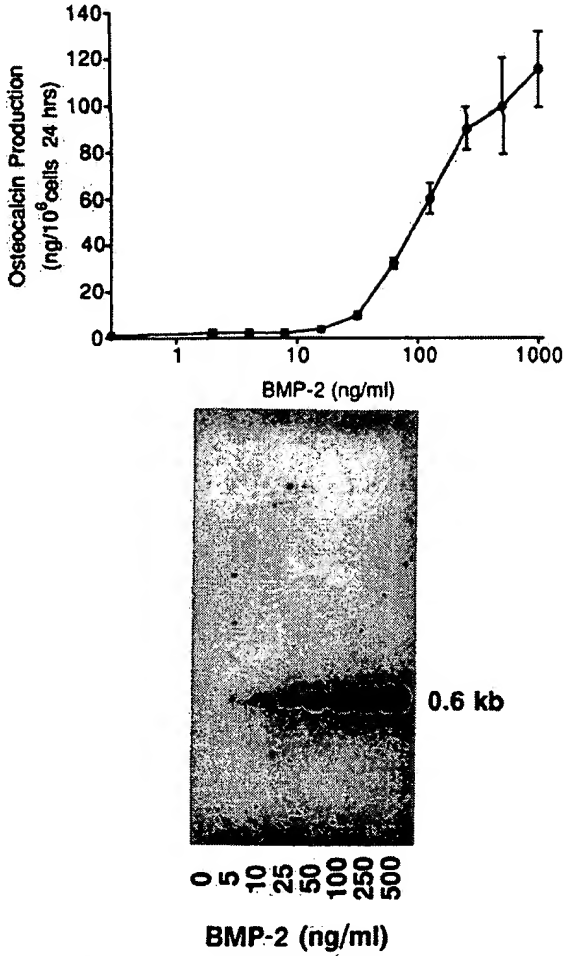

FIG. 7. Induction of osteocalcin mRNA (*lower panel*) and osteocalcin protein (*upper panel*) in W-20–17 cells treated for 4 days with or without rhBMP-2. A Northern blot analysis of poly(A)-containing RNA prepared from W-20–17 cells was performed. All lanes were loaded with equal amounts of total RNA. Secreted osteocalcin protein was measured in W-20–17-conditioned media by RIA. Results are expressed as the mean ± SEM of three experiments. Statistically significant levels of osteocalcin secretion were observed in W-20–17 cells treated with 31 ng/ml or more rhBMP-2 ($P < 0.05$). kb, Kilobases.

Discussion

In this report we demonstrate that rhBMP-2, which has been shown to induce bone formation *in vivo*, stimulated alkaline phosphatase activity in W-20–17 cells, a clonal cell line derived from mouse bone marrow stroma.

TABLE 2. Effects of osteoregulatory agents on the alkaline phosphatase activity of W-20-17 cells

Additions	Alkaline phosphatase activity (μmol NPP/h·mg protein)
None	3.8 ± 1.0
10^{-6} M dexamethasone	2.8 ± 0.7
10^{-6} M hydrocortisone	3.2 ± 0.7
10^{-8} M 1,25-$(OH)_2D_3$	2.6 ± 0.6
10^{-6} M PTH	2.6 ± 0.5
0.1 ng/ml TGFβ	2.4 ± 0.3
1.0 ng/ml TGFβ	2.0 ± 0.4[a]
10 ng/ml TGFβ	2.2 ± 0.5
50 ng/ml TGFβ	2.0 ± 0.4[a]
100 ng/ml BMP-2	10.2 ± 2.1[a]
10 ng/ml TGFβ + 100 ng/ml BMP-2	7.6 ± 1.9[a]
10^{-7} M retinoic acid	3.8 ± 0.6
500 ng/ml BMP-2	14.6 ± 1.4[a]
10^{-7} M retinoic acid + 500 ng/ml BMP-2	16.0 ± 0.8[a]
rhBMP-4[b]	9.0 ± 1.0[a]

Confluent W-20-17 cells were treated for 24 h and then assayed for alkaline phosphatase activity. The data represent the mean ± SEM of five different experiments. In each experiment, quadruplicate determinations were averaged.

[a] $P < 0.05$.

[b] A 1:4 dilution of conditioned media from CHO cells expressing human recombinant BMP-4. Conditioned media from mock-transfected CHO cells showed no stimulation (data not shown).

This effect was dose responsive, with significant stimulation beginning at 6 ng/ml rhBMP-2. However, maximal levels of stimulation were not achieved at concentrations expected to exceed the physiological dose range of a local regulatory factor. Moreover, rhBMP-2 has been reported to induce alkaline phosphatase activity in other cell lines at concentrations equal to or significantly higher than those reported here (18–20). Our experiments with rhBMP-2-conditioned media indicate that these results are not due to inactivation of the protein or loss of rhBMP-2 by excessive nonspecific binding. Therefore, the relative insensitivity of this biological response may simply reflect a low affinity BMP-2 receptor or cross-binding of BMP-2 to receptors for a related protein. The fact that TGFβ does not stimulate alkaline phosphatase activity in these cells indicates that rhBMP-2 is not mediating this response through TGFβ receptors in spite of the sequence similarity between these two factors. However, rhBMP-4 also stimulated alkaline phosphatase in W-20–17 cells. Therefore, while the relative efficacy of rhBMP-4 has not been determined in this experiment, the possibility remains that rhBMP-2 mediates the induction of alkaline phosphatase activity through binding to receptors for rhBMP-4 or some other BMP.

The stimulation of alkaline phosphatase activity in W-20–17 cells was not accompanied by a mitogenic effect of rhBMP-2, but did appear to require protein synthesis, since cycloheximide cotreatment effectively eliminated the stimulatory effect. The delayed time course of alkaline phosphatase induction is consistent with this requirement for protein synthesis and suggests that the rhBMP-2 effect is not accomplished by a direct activation of the enzyme. These findings are also consistent with a recent report demonstrating increased levels of alkaline phosphatase mRNA in C3H10T1/2 cells treated with rhBMP-2 (18).

Since induction of alkaline phosphatase activity has long been recognized as one marker of osteoblast differentiation, and W-20–17 cells are derived from bone marrow stroma, it was postulated that these cells might represent an osteoblast progenitor. Therefore, a number of other criteria associated with the osteoblast phenotype were investigated. Whereas some osteoblastic cells have been shown to mineralize *in vitro* and *in vivo* (13, 21–23), W-20–17 cells failed to mineralize under similar experimental conditions. Dexamethasone, hydrocortisone, 1,25-$(OH)_2D_3$, and PTH, agents that alter alkaline phosphatase activity in other osteoblastic cells (24–28), did not affect alkaline phosphatase activity in W-20–17 cells. However, when W-20–17 cells were treated with rhBMP-2, they exhibited a degree of PTH responsiveness similar to that of several other osteoblastic cell lines (14, 15, 20). Finally, W-20–17 cells treated with rhBMP-2 did synthesize osteocalcin, the only known bone-specific protein produced by osteoblasts (29). A similar effect of rhBMP-2 on osteocalcin expression has recently been observed in a postulated osteoprogenitor cell line derived from calvaria (20). These results collectively indicate that rhBMP-2 treatment of W-20–17 cells results in expression of some features unique to osteoblastic cells. The fact that osteocalcin was detected only after rhBMP-2 treatment suggests that W-20–17 cells may represent an osteoblast progenitor cell that can be induced by rhBMP-2 to differentiate toward the osteoblast phenotype.

Acknowledgments

We are grateful to Drs. S. Tsai and D. Nathan for providing oligoclonal W-20 cells. We also thank Dr. D. Israel, J. Nove, and K. Kerns for providing BMP-4-conditioned medium, and Drs. E. Wang and B. Foster for purified BMP-2.

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Bone morphogenetic protein 2 transiently enhances expression of a gene, Id (inhibitor of differentiation), encoding a helix–loop–helix molecule in osteoblast-like cells

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ABSTRACT **Bone morphogenetic protein 2 (BMP-2) is a potent inducer of differentiation of osteoblasts both *in vivo* and *in vitro*. We examined the action of BMP-2 on expression of a helix–loop–helix-type transcription factor, Id (inhibitor of differentiation), in osteoblast-like cells, as well as in osteoblast-enriched cells and possible precursor cells. To our surprise, BMP-2 enhanced Id gene expression in the cell types of osteoblastic lineage we examined. The maximal BMP-2 enhancement was observed within 24 hr in early proliferating cultures and the enhancement lasted up to 96 hr. The BMP-2 effect was not blocked by actinomycin D, while it was blocked by cycloheximide, suggesting that BMP-2 regulates Id gene expression at least in part via posttranscriptional events, which require protein synthesis. Other experiments indicated that BMP-2 did not further enhance Id mRNA levels promoted by dexamethasone, while BMP-2 did not resume the Id mRNA levels suppressed by 1,25-dihydroxyvitamin D_3. Similar BMP-2 enhancement of Id message expression was also observed in osteoblast-enriched fetal rat calvaria cells as well as C3H10T½ cells. These results indicate that BMP-2 enhances expression of Id in early cultures of osteoblastic cells and suggest that enhancement of Id expression may somehow be involved in the promotion of differentiation by this cytokine in these osteoblastic cells and in their precursor cells.**

The differentiation process of cells is a combination of multiple complex events. Upon certain stimuli, undifferentiated cells are induced to acquire specific phenotypes, mostly, if not completely, by expressing genes encoding a set of proteins unique to a certain type of cell (1). Osteoblasts are derived from so-called undifferentiated mesenchymal cells. This notion is based on the observations in embryonic or postfracture osteogenesis as well as an ectopic bone formation model. In the latter system, new bone formation can be induced by implanting demineralized bone matrices in muscle or subcutaneous tissues (2). Upon differentiation into osteoblasts, mesenchyme-derived cells express differentiation-related phenotypes such as a high level of alkaline phosphatase, receptors for parathyroid hormone, type I collagen, osteocalcin, osteonectin, osteopontin, and other noncollagenous bone matrix proteins (3). Expression of the multiple genes encoding these proteins can be differentially up- or down-regulated by many modulators such as steroid hormones, peptide hormones, cytokines, as well as autacoids (4).

A few molecules were recently reported to up-regulate the expression of most of these genes in undifferentiated fibroblast-like cells and hence induce osteoblastic differentiation (5, 6). Bone morphogenetic protein 2 (BMP-2) is one of these molecules, which was purified and molecularly cloned by Wozney *et al.* (7). BMP was first described by Urist (8) as a proteinaceous activity present in demineralized bone matrix, which, as mentioned above, induces ectopic bone formation. So far, at least eight BMPs are reported of which seven are members of the transforming growth factor type β (TGF-β) superfamily (9). Once such molecules have been identified, the next obvious step is to deduce the intracellular mechanism(s) through which BMP-2 induces osteoblastic differentiation. BMPs bind to distinct cell-surface receptors, whose sizes are similar to TGF-β receptors (10). Since receptors for both TGF-β (11) and activin (12, 13), another TGF-β family member, possess serine-threonine kinase domains as their intracellular structures, it is likely that a BMP receptor(s) might also have similar domains and kinase activities. Subsequent phosphorylation or dephosphorylation events would promote the expression of each individual phenotypic gene, and/or it might enhance a master regulatory gene(s) responsible for governing the expression of downstream phenotypic genes. Although it is not known whether any master regulatory gene(s) may exist in the osteoblast system, such molecules have been identified in myoblasts/myocytes, which, like osteoblasts, are derived from undifferentiated mesenchymal cells. One of the molecules identified is MyoD (14, 15), which converts fibroblasts into myoblasts when overexpressed by transfection (16). The MyoD subfamily belongs to a larger protein family with (basic) helix–loop–helix structures. Members of another subfamily of helix–loop–helix proteins lack basic regions in the domain, such as Id, and act as inhibitors of differentiation.

Id appears to be expressed ubiquitously (17), while the level of its expression is specifically regulated in differentiating cells and this control is critical for differentiation and development or modulation of the differentiation by hormones or other factors (18–20).

We previously reported (21, 22) that the Id gene is expressed in cells of the osteoblast lineages and that calcitropic hormones modulate the level of Id gene expression in these cells. To elucidate the mechanism of BMP action in osteoblastic differentiation, we examined BMP modulation of Id gene expression in these cells. Here we show that BMP up-regulates Id gene expression in osteoblastic cells at least in part via posttranscriptional control.

MATERIALS AND METHODS

Cells and Cell Culture. $MC3T3E_1$ cells were kindly provided by H. Kodama (Oh-U University) (23). The cells were grown in alpha-MEM supplemented with 5% fetal bovine serum (FBS) under an atmosphere of 5% CO_2/95% air at 37°C. C3H10T½ cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FBS under

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Abbreviations: BMP, bone morphogenetic protein; TGF-β, transforming growth factor type β.

5% CO_2/95% air at 37°C. Primary osteoblast-enriched cells were obtained by sequential collagenase digestion from 18-day fetal rat calvaria as described (24). The cells were grown in modified F-12 medium (25, 26) supplemented with 5% FBS under 5% CO_2/95% air at 37°C.

RNA Extraction and Northern Blot Analysis. Total RNA was extracted at the indicated time points according to the method described (27). Briefly, the cells were rinsed with phosphate-buffered saline three times, followed by extraction in 4 M guanidinium isothiocyanate, homogenized, and then extracted with acid phenol. The aqueous phase was combined with 1-propanol and the RNA was precipitated, rinsed with 75% ethanol, and resuspended in a buffer containing Tris·HCl (pH 7.4), 1 mM EDTA, and 0.1% SDS. The RNA was quantitated by spectrophotometry at 260 and 280 nm. Total RNA (10 or 20 μg) was fractionated by electrophoresis in 1% agarose/formaldehyde gels. Northern blot analysis was carried out as described (21). Id cDNA was kindly provided by Harold Weintraub.

Measurements of Alkaline Phosphatase Activity and DNA Content. Cells were plated in 2-cm^2 wells, rinsed twice with 0.9% NaCl, and lysed in an aliquot (500 μl) of a buffer (10 mM Tris·HCl, pH 7.5/0.5 mM $MgCl_2$/0.1% Triton X-100). The cell lysates were collected, sonicated twice for 15 sec each, and centrifuged at 12,000 × *g* for 10 min. The supernatants were used for measurement of alkaline phosphatase activity with *p*-nitrophenyl phosphate used as substrate (26), and DNA content was determined by the diaminobenzoic acid method (29).

RESULTS

Id mRNA is expressed as a 1.2-kb band in $MC3T3E_1$ cells and the level of expression was relatively high in proliferating cells in early cultures and then declined with time. Treatment with recombinant human BMP-2 (10 ng/ml) enhanced Id mRNA levels ≈3-fold within 24 hr (Fig. 1 *A* and *B*). Id mRNA levels were normalized against those of actin mRNA, which were not affected by BMP-2 treatment *per se*. BMP-2 enhancement of Id expression was maximal by 24 hr. As basal Id mRNA levels declined 4- to 5-fold by days 2 and 4 in control cells, the Id mRNA levels in the treated cells also declined to the levels similar to the control (Fig. 1 *A* and *B*). This BMP-2 effect on Id mRNA level was dose dependent as shown in Fig. 1*C*. The effect was observed as low as 1 ng/ml and was still increasing at 100 ng/ml (data not shown). BMP-2 enhancement of Id expression was unexpected since enhancement of Id expression has been shown to be associated with inhibition of differentiation in many other systems (18, 19). Therefore, we examined whether BMP-2 suppresses expression of the osteoblastic phenotypes in our system. We observed that BMP-2 enhanced osteopontin mRNA expression ≈3-fold in these $MC3T3E_1$ cells (T.O. and M.N., unpublished data). BMP-2 treatment also enhanced expression of another osteoblastic phenotype, alkaline phosphatase (data not shown), as reported previously (28) in $MC3T3E_1$ cells.

To examine the level at which BMP-2 regulates Id gene expression, BMP-2 treatment was conducted in the presence of actinomycin D or cycloheximide. Treatment with actinomycin D by itself elevated the Id mRNA level ≈2-fold. However, BMP-2 further enhanced by 2-fold the Id mRNA level in the presence of actinomycin D, while the BMP-2 effect was blocked by the presence of cycloheximide (Fig. 2).

The actions of local regulators are under the influence of systemic hormones. Therefore, we examined whether BMP-2 action is modulated by hormones that are involved in regulation of calcium metabolism. Treatment with 10 nM 1.25-dihydroxyvitamin D_3 (vitamin D_3) suppressed Id mRNA levels, and simultaneous treatment with BMP-2 (100 ng/ml) did not counteract the decline of Id mRNA abundance by vitamin D_3. Treatment with dexamethasone enhanced Id mRNA levels as observed previously, but BMP-2 did not further enhance the level of Id mRNA expression by dexamethasone (Fig. 3).

Since $MC3T3E_1$ cells may represent only a certain type of osteoblast, we wondered whether the BMP-2 effect on Id could be a general phenomenon or something specific to these cells. We therefore examined the effect of BMP-2 on Id mRNA expression in cells other than $MC3T3E_1$ cells. In the fibroblastic cell line C3H10T½, treatment with BMP-2 enhanced Id mRNA expression to an extent similar to that observed in $MC3T3E_1$ cells. In these cells, vitamin D_3 treatment suppressed Id mRNA expression, and this was not resumed by concurrent treatment with BMP-2 as observed in $MC3T3E_1$ cells. Also similar to $MC3T3E_1$ cells, dexamethasone enhanced Id mRNA expression, while concurrent treatment with BMP-2 did not further enhance Id mRNA levels. Collectively, these effects were similar to those observed in $MC3T3E_1$ cells (Fig. 4) with identical treatments.

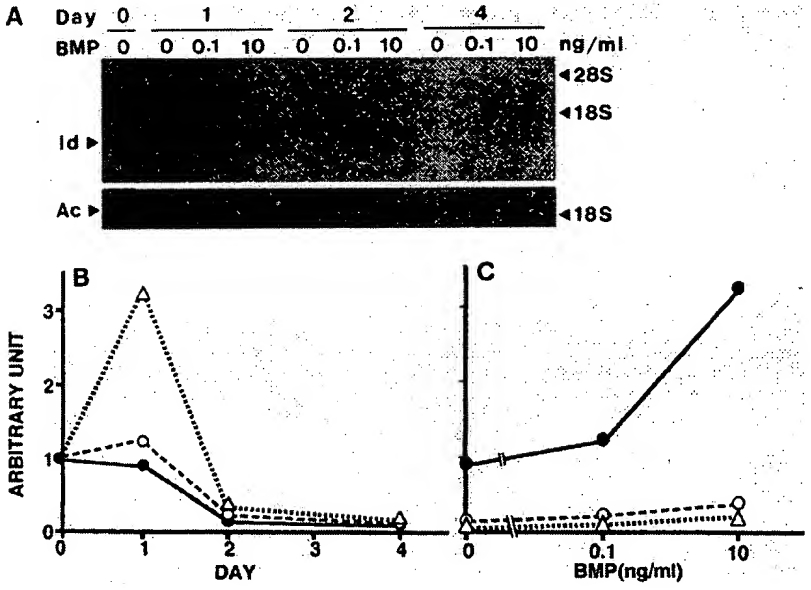

FIG. 1. Effect of BMP-2 on Id mRNA expression. $MC3T3E_1$ cells were plated at 2000 cells per cm^2 and cultured in alpha-MEM supplemented with 5% FBS. After 48 hr, the medium was replaced and BMP-2 was added at the indicated concentrations. RNA was extracted at the indicated time points after addition of BMP-2 and 10 μg was loaded on each lane. (*A*) Northern blot analysis. Positions for Id, actin (Ac), and 28S and 18S RNA are indicated. (*B*) Quantitation of Northern blot analysis shown in *A*. Time course at each dose of BMP-2. Id mRNA bands were quantitated by densitometry and data are normalized against corresponding actin mRNA level. ●, 0 ng/ml; ○, 0.1 ng/ml; △, 10 ng/ml. (*C*) Quantitation of Northern blot analysis shown in *A*. Dose dependence at each time point is shown. ●, day 1; ○, day 2; △, day 4.

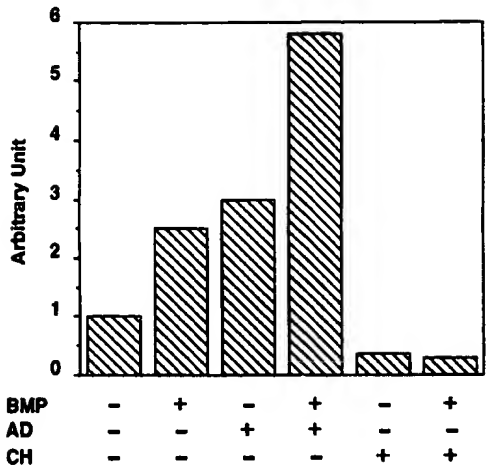

FIG. 2. Effect of actinomycin D (AD) or cycloheximide (CH) on Id message enhancement by BMP-2. MC3T3E$_1$ cells were plated as described in the legend to Fig. 1. After 48 hr, the medium was changed and cells were cultured in the presence or absence of AD (0.2 μg/ml), CH (2 μg/ml), BMP-2 (100 ng/ml), or combinations as indicated. Inhibitors were added 15 min prior to addition of BMP-2. RNA was extracted 24 hr later and analyzed as described in the legend to Fig. 1. Quantitation of Northern blot analysis is shown. Data were estimated as described in the legend to Fig. 1.

Finally, we examined whether BMP-2 acts similarly in primary cultures of normal osteoblastic cells. In fetal rat calvaria-derived osteoblast-enriched cells, Id mRNA was also expressed constitutively, and dexamethasone and BMP-2 enhanced Id mRNA levels to an extent similar to that observed in MC3T3E$_1$ cells (Fig. 5).

DISCUSSION

In this study, we have shown that recombinant human BMP-2 enhances the level of Id mRNA in osteoblastic cells. This BMP-2 effect was maximal in early cultures of these cells, where the cells are proliferating. Induction of cartilage or

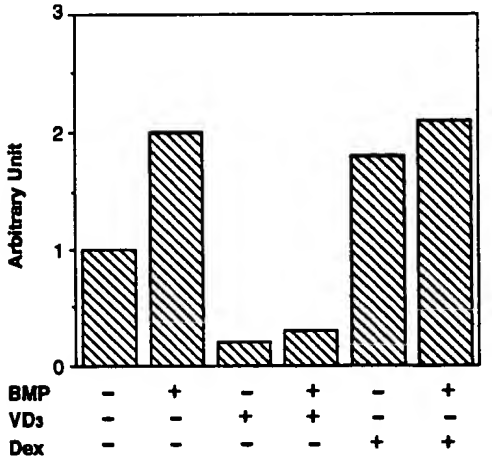

FIG. 3. Modulation by vitamin D$_3$ (VD$_3$) or dexamethasone (Dex) of BMP-2 enhancement of Id mRNA expression. MC3T3E$_1$ cells were plated and cultured as described in the legend to Fig. 1. Medium was changed 48 hr after plating. Cells were treated for 24 hr with BMP-2 (100 ng/ml), VD$_3$ (10 nM), and Dex (100 nM) alone or in the combination indicated. Northern blot analysis was conducted as described in the legend to Fig. 1. Quantitation of Northern blot analysis is shown. Data were estimated as described in the legend to Fig. 1.

FIG. 4. Effect of BMP-2 on Id mRNA expression in C3H10T½ cells. C3H10T½ cells were plated at 2000 cells per cm^2 and cultured in DMEM supplemented with 5% FBS. After 48 hr, the medium was changed and cells were treated for 24 hr with BMP-2 (100 ng/ml), vitamin D$_3$ (VD$_3$) (10 nM), or dexamethasone (Dex) (100 nM) alone or in the combination indicated. Northern blot analysis using 10 μg of RNA per lane was carried out as described in the text. Positions for Id, actin (Ac), and 28S and 18S RNA are indicated.

bone formation by BMP-2 *in vivo* suggests that BMP-2 induces differentiation of undifferentiated progenitor cells to chondrocytes and/or osteoblasts. BMP-2 has been shown to enhance expression of phenotypic markers of these types of cells, such as alkaline phosphatase in undifferentiated C3H10T½ cells (30) as well as several relatively undifferentiated cell types (5, 6). We have also observed (T.O. and M.N., unpublished data) that treatment with BMP-2 enhances osteopontin gene expression in MC3T3E$_1$ cells. We and others described previously that the level of Id expression in the osteoblast-like cell MC3T3E$_1$ significantly decreases in late cultures (31, 32), which suggested the presence of a reciprocal relationship between levels of Id expression and stages of differentiation. Transfection of Id expression vector was also reported to suppress alkaline phosphatase expression (32). It is intriguing that Id expression is upregulated in early cultures by the reagent BMP-2, which enhances differentiation in later cultures. The enhancement of Id expression in proliferating MC3T3E$_1$ cells in early cultures may coincide with the observation that BMP-2 stimulates DNA synthesis in some cells of osteoblastic lineage. On the other hand, BMP-2 enhancement of differentiation-related osteoblast phenotypes appears to require the preceding decline of Id level as a prerequisite condition since at least some differentiation markers such as alkaline phosphatase activity are enhanced by BMP-2 in confluent cells. One of several other possibilities for this paradoxical phenomenon may be that the cells have to undergo transient and mild dedifferentiation prior to further differentiation. It has been observed in the regeneration process in injured skin that epidermal cells in the basal layer first dedifferentiate before they redifferentiate into hair follicle cells or sweat gland cells, which are lost by the trauma. It may also be possible that Id has functions other than negative regulation of differentiation

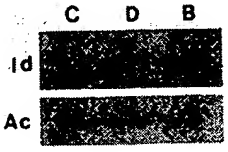

FIG. 5. Effect of BMP-2 on Id mRNA expression in primary cultures of osteoblast-enriched rat fetal calvaria cells. Primary osteoblast-enriched cells from rat calvaria were prepared as described. Cells were treated for 24 hr with dexamethasone (lane D) (100 nM) or BMP-2 (lane B) (100 ng/ml). Lane C, control. Northern blot analysis using 10 μg of RNA per lane was carried out as described. Positions for Id and actin (Ac) are indicated. Ratios of Id mRNA over Ac mRNA level are 1.0 (control), 1.6 (dexamethasone), and 3.0 (BMP-2).

and BMP-2 may not only induce differentiation of some types of cells but also inhibit differentiation of other types, as seen in some cells (5).

We observed that actinomycin D treatment by itself enhanced the basal level of Id mRNA expression ≈2-fold, suggesting that the blocking of transcription of some suppressor factor(s) causes increased expression of Id mRNA levels. Even in the presence of actinomycin D, the effect of BMP-2 was not blocked and further enhanced Id mRNA level, indicating that BMP-2 regulates Id gene expression through posttranscriptional events. This regulation requires new protein synthesis since BMP-2 action was blocked by cycloheximide.

We reported previously that vitamin D_3 and dexamethasone, which modulate the differentiation of osteoblastic cells, down- and up-regulated Id mRNA expression, respectively. In cotreatment experiments, BMP-2 enhancement of Id mRNA level was counteracted by vitamin D_3. BMP-2 did not further enhance Id mRNA levels more than those promoted by dexamethasone. In our preliminary experiments, the dexamethasone effect on Id was not blocked by actinomycin D. This may suggest that dexamethasone and BMP-2 share a certain pathway(s) in enhancing Id mRNA abundance at the posttranscriptional level. These results may reflect an orderly relationship between the effects of BMP-2 and systemic regulatory hormones in controlling Id mRNA expression.

We observed that BMP-2 also up-regulates Id mRNA expression in C3H10T½ cells and rat calvaria-derived osteoblast-enriched cells in which BMP-2 either induces or enhances differentiation-related phenotypic expression. This finding indicated that the BMP-2 effect on Id expression is not confined to the particular osteoblast-like cell line $MC3T3E_1$, but it is a more general phenomenon, which is at least observed in normal osteoblast-enriched cells, as well as undifferentiated precursor-like cells.

The enhancement of Id, an inhibitor of differentiation, by the potent stimulator of osteoblastic differentiation reveals unusual aspects of these molecules. BMP-2 is expressed in many tissues other than bone (brain, kidney, etc.) and also affects development of dorsoventral pattern by acting at the very early stage of *Xenopus* embryos. Id and other helix–loop–helix molecules may be involved in the various aspects of morphogenesis, which is under the control of the BMPs.

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B96

ASCORBIC ACID DEFICIENCY INHIBITS INTEGRIN EXPRESSION PRIOR TO ITS EFFECT ON COLLAGEN SYNTHESIS IN FETAL RAT PARIETAL BONE CULTURES. D. Gania*, G. Pastizzo*, M.B. McCarthy and G. Gronowicz. Dept. of Orthopaedics, University of Connecticut, Farmington, CT.

Ascorbic acid deficiency results in underhydroxylation of collagen and an inhibition of collagen synthesis. We investigated the relationship between collagen synthesis and the β_1 integrin subunit of the collagen receptor. Since the α subunit of the collagen receptor in bone has not been conclusively identified, the β_1 was studied. Treatment of 20 day fetal rat parietal bones with 0, 1, 10 and 100 µg/ml of ascorbic acid for 96 hours had no significant effect on collagen synthesis as determined by [^{3}H]proline incorporation into collagenase digestible protein and noncollagen proteins. No significant differences were found in dry weight, DNA synthesis or DNA content. However, significant effects were demonstrated in calcium and hydroxyproline content.

Ascorbic acid (µg/ml)	Calcium (µg/bone)	Hydroxyproline (µg/bone)
100	17.6±0.8	41.0±2.0
10	16.5±0.8	17.3±3.8*
1	11.3±0.5*	20.5±6.5*
0	12.3±0.7*	9.8 ±3.5*

(*=p<0.05 compared to 100 µg/ml)

Light microscopy demonstrated that preosteoblasts and osteoblasts were swollen and no longer contiguous in bones treated with 0, 1 and 10 µg/ml ascorbic acid compared to control bones treated with 100 µg/ml. The osteoid seam was wider in the ascorbic acid-deficient bones. Immunofluorescence staining for the β_1 integrin was found primarily in the osteoblast layer overlying the bone in controls. Staining was markedly diminished in a dose-dependent manner in bones treated with 0, 1 and 10 µg/ml ascorbic acid.

These results demonstrate that Type I collagen synthesis and the collagen integrin are independently regulated in bone culture. In addition, integrins may be able to detect the conformation of collagen fibrils in the matrix and alter receptors on the cell surface accordingly.

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SEQUENCES THAT MEDIATE THE INDUCTION OF OSTEOCALCIN GENE TRANSCRIPTION BY rhBMP-2. K.Goto, V.Rosen, J.M.Wozney, H.M. Kronenberg and M.B. Demay. Endocrine Unit, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114 and Genetics Institute Inc., Cambridge, MA 02140.

rhBMP-2 induces the differentiation of mesenchymal cells into osteoblastic cells which express the osteoblast-specific protein osteocalcin. Identification of the sequences in the osteocalcin gene responsible for this induction will permit the characterization of factors involved in the developmental regulation of osteocalcin gene expression and in osteoblast differentiation. It has previously been shown that osteocalcin-CAT fusion gene expression can be induced by rhBMP-2 in transient gene expression assays in MLB13MYC clone 17(C17) cells. The DNA sequences from -1750 to +84 are sufficient for this induction. Deletion of the sequences between -522 and -428 results in the abolition of responsiveness to rhBMP-2. To further characterize this region, osteocalcin-CAT fusion genes containing the sequences from -522 to -455, -522 to -341 and -458 to -403 were ligated into an osteocalcin promoter-CAT fusion gene and transfected into C17 cells. After 72 hours of treatment with 250ng/ml of rhBMP-2, CAT activity was induced 2.0-fold in fusion genes containing the sequences from -522 to -341, and -458 to -403. No induction was observed in fusion genes containing the sequences from -522 to -455.

To determine whether rhBMP-2 induced proteins which interacted with these sequences, nuclear extracts were prepared from untreated C17 cells and C17 cells treated with 100ng/ml of rhBMP-2 for 72 hours. When the sequences from -458 to -403 were labeled and incubated with these extracts, unique protein-DNA complexes were observed in the extracts prepared from rhBMP-2 treated cells. The major retarded signal was abolished with 10-fold molar excess of oligonucleotides containing the sequences from -430 to -403, -458 to -418, and -458 to -410, but not with the sequences from -458 to -433 or the 1,25(OH)$_2$D3 response element (-458 to -442).

These data demonstrate that the sequences from -458 to -403 are sufficient for rhBMP-2 induction. Furthermore, rhBMP-2 induces a protein which binds to the sequences in this oligonucleotide, downstream from the 1,25(OH)$_2$D3 receptor binding site.

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CHARACTERIZATION OF THE MAJOR NON-COLLAGENOUS PROTEINS IN CHICKEN BONE: IDENTIFICATION OF A NOVEL 60 kDa ACIDIC PROTEIN. **Y. Gotoh, M.J. Glimcher and L.C. Gerstenfeld. Laboratory for the Study of Skeletal Disorders and Rehabilitation, Children's Hospital and Harvard Medical School, Boston, MA 02115**

In order to isolate and characterize the major non-collagenous proteins of chicken bone, a serial extraction procedure was developed by which bone powder was sequentially treated by 4M guanidine HCl (G), followed sequentially by 0.3 M HCl (H), neutral high salt 1M NaCl (NS), and 4M guanidine HCl (NG). This procedure specifically extracted proteins of differing solubility such that osteocalcin (OC) was solubilized in H>NS; osteopontin (OPN) was exclusively extracted in H; α2 HS glycoprotein was extracted in NS>H; bone sialoprotein (BSP) was extracted in NS>H; osteonectin (ON) was extracted in NS>NG, however it was not soluble at all in acidic conditions; and collagen was extracted in NS>NG>H. Amino terminal sequence analysis was performed to identify each of these proteins. Additional internal sequence was obtained for ON demonstrating that the avian form of this protein is over 80% conserved for the available sequence obtained for the 10% of the total sequence presently available. During the course of these studies, a novel ~60 kDa protein was identified showing a unique NH_2 terminal sequence D D P S F D S L G G R H S E G T S. This protein has a high acidic amino acid composition and amino acid analysis indicated that it was similar in composition to other acidic glycoproteins in bone. However, it contained no cysteine and had a lower asp than glu content. By immunological reactivity, it was not recognized by polyclonal antibodies to either BSP or OPN and did not react with a monoclonal antibody to decorin. This protein was not present in chicken serum based on negative reactivity with a polyclonal antibody directed to itself and no comparable sequence was found in the GenBank or EMBL protein sequence database. In summary, these data provide the first sequence for a non-mammalian form of ON and identify a novel acidic bone protein.

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BONE MARROW DEVELOPMENT AND ITS RELATIONSHIP TO BONE FORMATION IN VIVO: A histological study using an implantable titanium device in rabbits. H. Zhou*, [1]P. Choong*, [2]S.T. Chou*, [1]P. Aspenberg*, T.J. Martin, and K.W. Ng. Department of Medicine, The University of Melbourne, St Vincent's Hospital, Fitzroy, Victoria, 3065, Australia. [1]Lund University Hospital, S-221 85 Lund, Sweden. [2]Repatriation General Hospital, Heidelberg, Victoria 3081, Australia.

During embryogenesis, the creation of marrow sinusoids results from the coupled processes of osteogenesis and osteoclastic resorption. Influx of marrow cellular elements follow the formation of bone during endochondral as well as intramembranous ossification.

We set out to further define the relationship between marrow development and bone formation by implanting an intraosseous titanium device into the tibiae of rabbits. A hollow channel is incorporated into the device into which tissue can grow, and the histological sequence of events was observed over 7 weeks.

The channel was in direct continuity with the marrow cavity and isolated from the endosteum. Therefore, immediate marrow regeneration was expected to follow dissolution of the blood clot. Instead at 2W, the first rod-shaped piece of histological tissue consisted of spindle-shaped cells in the centre, flanked at both ends by islands of *osseous tissue* including osteoblasts and osteoclasts. Ingrowth of bone reached the center of the specimen by 4W. However, osteoclastic resorption continued unabated and the quantity of trabecular bone began to diminish so that by 7W, only a thin layer of cortical bone remained. Beginning at 3W, neocapillaries became visible in the intertrabecular spaces. The marrow cavity progressively enlarged with time to be populated by hemopoietic elements.

Although the opportunity existed for immediate marrow regeneration, formation of a marrow cavity and its hemopoietic elements was still preceded by bone formation. Our results provide strong evidence for the primacy of bone formation over marrow development.

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Osteogenic Protein-1 (BMP-7) Inhibits Cell Proliferation and Stimulates the Expression of Markers Characteristic of Osteoblast Phenotype in Rat Osteosarcoma (17/2.8) Cells

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We recently showed that osteogenic protein-1(OP-1), a bone morphogenetic protein member of TGF-β superfamily, induces endochondral bone formation *in vivo*, and stimulates growth and differentiation of osteoblasts in rat calvarial-derived cell cultures. In the present study, we examined the effect of OP-1 on cell growth and expression of markers that are characteristic of osteoblast phenotype using the clonal rat osteosarcoma cells (ROS 17/2.8). A comparison of OP-1 and TGF-β1 effects on cell growth showed that, both OP-1 and TGF-β1 inhibited DNA synthesis up to 90 percent and 60 percent of the controls at concentrations of 10 ng/ml and 1 ng/ml, respectively, in serum-free medium. In the presence of 5% serum, TGF-β1 did not have any significant inhibitory effects while 40 ng OP-1/ml inhibited the DNA synthesis up to 80% of the controls. Examination of collagen synthesis showed that 40 ng OP-1/ml increased the expression of type I collagen mRNA, and thus increased collagen synthesis (4-fold), as examined by collagenase-digestible protein. Evaluation of markers that are characteristic of the osteoblast phenotype demonstrated that OP-1 stimulated cAMP production in response to PTH (10-fold at 200 ng/ml), alkaline phosphatase specific activity (ALPase) (4-fold at 80 ng/ml), and osteocalcin (OC) synthesis (4.5-fold at 40 ng/ml). Northern blot analysis revealed that OP-1 increased mRNA expression for both ALPase and OC in a dose-dependent manner. These data collectively demonstrate that OP-1 suppresses cell proliferation and stimulates the expression of markers characteristic of osteoblast phenotype in rat clonal osteoblastic osteosarcoma cells (ROS 17/2.8).

KEYWORDS: bone morphogenetic proteins, bone formation, osteoblasts, bone matrix proteins

INTRODUCTION

Employing the rat subcutaneous bone induction assay (Sampath and Reddi, 1981, 1983), several bone inducing proteins have recently been isolated from bovine demineralized bone matrix (Wong et al., 1988; Luyten et al., 1989; Sampath et al., 1990), which permitted the cloning of corresponding full length cDNAs and the related genes. These potent osteoinductive proteins are called bone morphogenetic proteins, BMP-2 to 6 (Wozney et al., 1988; Celeste et al., 1990) and/or osteogenic proteins, OP-1, OP-2 (Ozkaynak et al., 1990, 1992). The predicted amino acid sequences have revealed that these gene products are members of the TGF-β superfamily, all of which share a high degree of homology within the characteristic COOH-terminal 7 cysteine domain.

We demonstrated that a highly purified bovine osteogenic protein preparation contained dimers of OP-1 and BMP-2, and entertained the possibility that both homo- and heterodimers occurred in such preparations (Sampath et al., 1990). Human osteogenic protein-1, (OP-1, also called BMP-7) was produced recombinantly as a processed, disulfide-linked homodimer, and was shown to induce new

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bone formation with a specific activity equivalent to that of naturally purified bovine osteogenic protein, using the rat subcutaneous bone induction model (Sampath et al., 1992). In addition to bone induction, OP-1 was able to stimulate growth and expression of markers characteristic of the osteoblast phenotype in rat primary osteoblast-enriched cultures (Sampath et al., 1992). As osteoblast-enriched cultures obtained from rat calvaria contain both osteoblast progenitors and osteoblasts at various stages of differentiation, it has always been difficult to precisely characterize the population of cells responding to OP-1. In the present study, we therefore examined the effect of OP-1 on the clonal rat osteosarcoma cell line ROS 17/2.8 with respect to cell growth and expression of markers that are characteristic of osteoblasts. ROS 17/2.8 cells have been used widely to evaluate osteoblastic response to growth factors and hormones, as they express many phenotypes that are characteristics of mature osteoblasts (alkaline phosphatase activity, PTH responsiveness, and osteocalcin production). Here we present evidence with a thoroughly characterized clonal osteogenic cell line, that OP-1 inhibits cell proliferation and stimulates the expression of osteoblast phenotypic markers in ROS 17/2.8 cells.

MATERIALS AND METHODS

Cell Culture

ROS 17/2.8 rat osteosarcoma cells were generously provided by Dr Robert J. Majeska (Mount Sinai Hospital, NY). Cells were maintained in F-12 medium (GIBCO) containing 14 mM HEPES, 2.5 mM L-glutamine, 1.1 mM $CaCl_2$, supplemented with 5% heat inactivated fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). The cells were routinely subcultured twice a week. Cells were plated in 24-multiwell plates (Falcon Labware, Lincoln Park, NJ) or 48-well tissue culture plates (Costar Corp., Cambridge, MA) at varying cell densities as described in the text. Cells were cultured at 37°C, in 5% CO_2, and 88% humidity for 3–5 days without changing the medium until confluence (approximately 5×10^4 cells/cm^2); medium was then replaced with serum-free medium for 24 h prior to the addition of human OP-1 (produced recombinantly as described by Sampath et al., 1992) or TGF-β1 (R&D Systems, Minneapolis, MN).

To examine effects of OP-1 on osteocalcin synthesis, the medium was supplemented with 10% FBS. On day 2, cells were fed with fresh medium supplemented with fresh 10 mM β-glycerophosphate (Sigma). Beginning on day 5 and at twice weekly intervals, cells were fed with complete mineralization medium containing all of the above components plus fresh L(+)-ascorbate at a final concentration of 50 μg/ml (Sampath et al., 1992). Purified recombinant hOP-1 was stored at −20°C in 50% acetonitrile containing 0.1% trifluroacetic and added to the tissue culture wells at ≤5 μl/ml of medium with thorough mixing; control wells received the solvent vehicle only. Conditioned medium samples were diluted 1:1 in a radioimmunoassay buffer containing protease inhibitors (Gundberg et al., 1984) and stored at −20°C until assayed for osteocalcin. Cultures used for Northern blot analysis cells were plated into 100 mm petridishes (Corning) at a density of 10^4/cm^2 in the presence of 10% FBS. After 3–5 days of incubation, by which time the cells were confluent, the medium was replaced with medium containing 1% FBS. One day later, various doses of OP-1 were added and the cultures were incubated for an additional 5 days, with the replacement of medium and OP-1 after 3 days.

Cell Growth

The effect of OP-1 on the growth of ROS was examined by determining the rate of [^{3}H]-thymidine incorporation into total acid-insoluble DNA and by quantitating cell number. DNA synthesis rates were determined in triplicate cultures after 24 h of OP-1 treatment by adding [^{3}H]-thymidine (2 μCi/ml, 80 Ci/mmol; Du Pont-New England Nuclear) for 6 h before terminating the culture. Incorporation was terminated by aspirating the medium, and washing thrice with phosphate-buffered saline; the trichloroacetic acid (10%) precipitated radioactive DNA was extracted with 1.0% (w/v) sodium dodecyl sulfate and quantitated by liquid scintillation.

Collagen Synthesis

The rate of collagenous and noncollagenous protein synthesis was measured by pulse labelling with 25 μCi/ml [2,4-^{3}H]proline (Du Pont-New England Nuclear) for the last 6 h of culture. Various concentrations of OP-1 were added to triplicate confluent cultures in 24-well plates containing serum-free

medium. Following incubation, the cell layers were lysed by 3 freeze-thaw cycles and extracted with 1 M NaCl, Tris-HCl buffer, pH 7.4, containing 10 mM N-ethylmaleimide, 5 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride. Proteins from both the cell culture media and the cell lysates were precipitated with 10% trichloroacetic acid on ice; pellets were washed with acetone:ether (3:1 v/v), dried, resolubilized in 0.5 M acetic acid, and neutralized with NaOH. The amount of [^{3}H]proline incorporated into collagenase-digestible protein and nondigestible noncollagenous protein was determined as described by Peterkofsky and Diegelmann. The percent collagen synthesis was calculated after correcting for the higher relative abundance of proline in collagenase digestible protein (non collagenous protein values were multiplied by 5.4).

Alkaline Phosphatase Activity

Alkaline phosphatase specific activity was determined by the method of Reddi and Huggins (1972). Following removal of culture medium, cell layers were washed once with phosphate buffered saline (PBS) and sonicated in 500 µl of extraction buffer (0.15 M NaCl, 3 mM $NaHCO_3$, pH 7.4, containing 1% Triton X-100). Recovered samples (10 µl) were assayed for enzyme activity in 96-well plates with p-nitrophenyl phosphate (Sigma) as a substrate in 0.05 M glycine-NaOH buffer, pH 9.3, in a total volume of 100 µl; after 30 min at 37°C the reaction was stopped with 100 µl of 0.1 M NaOH and absorbance was measured at 400 nm on a Dynatech MR700 plate reader with p-nitrophenol as a standard. Protein concentration in each extract was measured by BIO-RAD. Results are presented in units/µg of protein, where 1 unit = 1 nmol of p-nitrophenol liberated per 30 min at 37°C.

3',5' cAMP Production in Response to PTH

To determine the cAMP production in the presence of PTH, cells were preincubated for 20 min with Ham's F12 medium containing 0.5% bovine serum albumin and 1 mM 3-isobutyl-1-methylxanthine (Sigma), and then 200 ng/ml of human PTH(1-34)(Sigma) was added and incubation continued for 8 min. The cell layers were solubilized in 1% Triton X-100, and the concentration of cAMP in the cell layer was determined using a cAMP kit (Amersham).

Osteocalcin Radioimmunoassay

Rat osteocalcin levels in the cell culture supernatant and cell-associated extracellular matrix were determined by a 3-day nonequilibrium radioimmunoassay as described previously (Gundberg et al., 1984), employing goat anti-rat osteocalcin (first antibody) and donkey anti goat IgG (second antibody). Data are reported as total ng of osteocalcin/culture. Osteocalcin associated with the extracellular matrix was quantitated by extracting PBS-washed cell layers with 0.5 ml of 0.5 M EDTA containing protease inhibitors and diluting appropriately for radioimmunoassay.

Northern Blot Analysis

For Northern blot analysis, total cellular RNA was prepared by the acid guanidine thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). 10 µg of total RNA was separated by electrophoresis on 1% agarose gels containing formaldehyde and transferred to nylon membrane filters (Nytran, Schleicher & Schuell, Keene, NH) by capillary blotting. Hybridizations were carried out for 16 h at 45°C with buffer containing 50% formamide, 5× SSPE, 5× Denhardt's solution, 1% sodium dodecyl sulphate (SDS), 25 µg/ml of salmon sperm DNA, 10% dextran sulphate, and the desired cDNA probe labelled with [α^{32}P]-dCTP by random priming (Kit; Boehringer Mannheim, Indianapolis, IN). After washing the filters, autoradiograms were made at −80°C by exposure to Kodak XAR-5 film (Kodak, Rochester, NY).

RESULTS

Cell Growth

In serum free medium, both OP-1 and TGF-β1 inhibited DNA synthesis in a dose-dependent manner (Fig. 1A). At 10 ng/ml, OP-1 inhibited DNA synthesis up to 90% of the control, while 1 ng/ml of TGF-β1 induced a maximum of 50% inhibition of the control. However, in the presence of 5% serum, TGF-β1 had only a negligible effect on DNA synthesis (maximum of 12% inhibition at 0.1 ng/ml), while OP-1 had 85% inhibition of the control at 100 ng/ml (Fig. 1B).

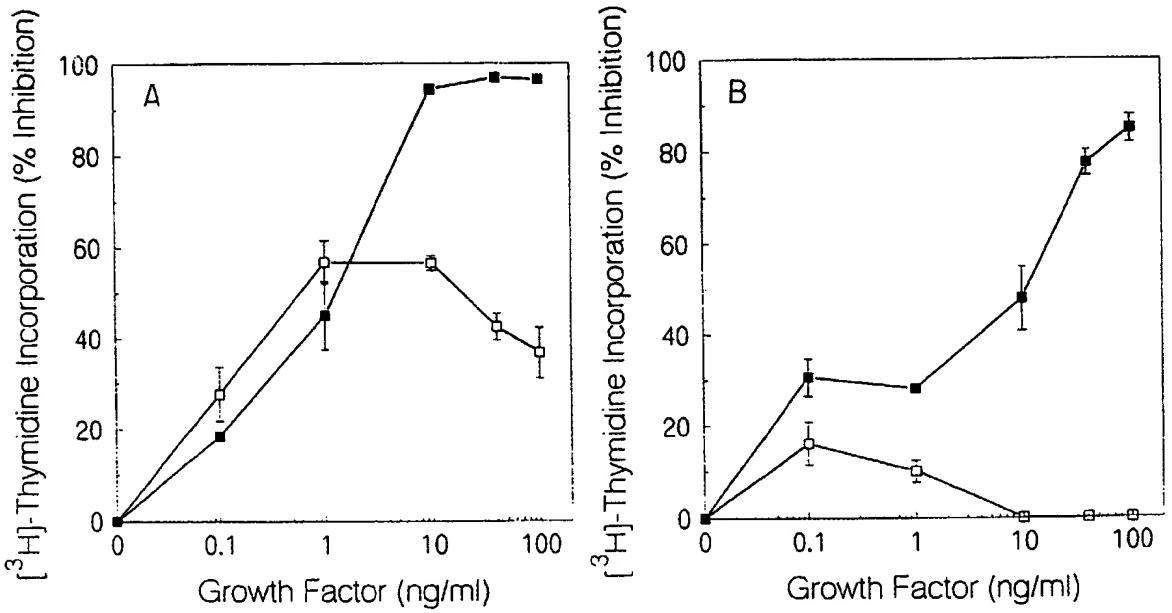

FIGURE 1. Effect of OP-1 and TGF-β1 on DNA synthesis in rat osteosarcoma 17/2.8 cells. ROS 17/2.8 cells were incubated in 48-well plates in serum-free medium (panel A) or in 5% serum-containing medium (panel B) for 24 h and treated with OP-1 (■), or TGF-β1 (□). After 18 h, the cultures were labelled with 2.5 μCi/ml of [^{3}H-] thymidine for 6 h. The incorporation of TCA insoluble [^{3}H] into cells was analyzed by solubilization of cells in SDS. The values are means ±S.D. of triplicate cultures.

Collagen Synthesis

OP-1 enhanced both the total amount of collagen synthesis and collagen synthesis relative to the synthesis of noncollagenous proteins in a dose-dependent manner. The results indicate that OP-1 at 100 ng/ml caused a maximum 4-fold increase in the total collagen synthesis (Fig. 2A). Furthermore, Northern blot analysis of OP-1 treated cultures showed a dose-dependent increase in the type I collagen mRNA levels (2-fold) at 40 ng/ml (Fig. 2B).

Expression of Osteoblast Phenotypic Properties

OP-1 treatment of ROS cells cultures at varying concentrations for 72 h resulted in a dose-dependent increase in alkaline phosphatase specific activity. OP-1 effects were seen at concentrations as low as 0.1 ng/ml and were maximal at 80 ng/ml, causing a 4-fold increase in the activity (Fig. 3A). TGF-β1, on the other hand, showed a modest 1.5-fold increase in alkaline phosphatase specific activity at 20 ng/ml. Long term culture of ROS 17/2.8 cells treated with OP-1 40 ng/ml for up to 10 days in the presence of ascorbic acid andβ-glycerophosphate exhibited the maximum alkaline phosphatase activity on day 7 (data not shown). Northern blot analysis of cultures treated with OP-1 for 7 days showed a dose-dependent increase in alkaline phosphatase mRNA expression, with a maximum increase at 40 ng/ml (Fig. 3B). The combined effect of OP-1 and TGF-β1 was examined by adding these factors together, incubating for 72 h, and assaying the cell extracts for ALPase. The results showed that OP-1 and TGF-β1 individually stimulated alkaline phosphatase production 6.5 fold (by 200 ng OP-1/ml) and 3-fold (by 4 ng TGF-β1/ml). When OP-1 and TGF-β1 were added together, the ALPase production was increased 9-fold, (200 ng/ml OP-1 and 4 ng/ml TGF-β1), thus showing an additive effect (data not shown).

Untreated ROS cells produced a small amount of cAMP in response to PTH, but the treatment with OP-1 increased the PTH-mediated cAMP production in a dose-dependent manner with a maximum 12-fold increase at 1 μg/ml of OP-1 (Table 1).

Effects of OP-1 on osteocalcin production were examined by radioimmunoassay of conditioned medium and cell lysates. The cultures were treated with 40 ng/ml of OP-1 with and without ascorbic acid and β-glycerophosphate, and samples were

analyzed on days 4, 7 and 10. OP-1 increased the osteocalcin production a maximum of 4.5-fold compared to the control on day 7 (Fig. 4A). Osteocalcin mRNA expression was also examined on cultures treated with various doses of OP-1 for 7 days. The results showed a dose-dependent increase in mRNA expression with a maximum of 1.7-fold increase at 100 ng/ml (Fig. 4B).

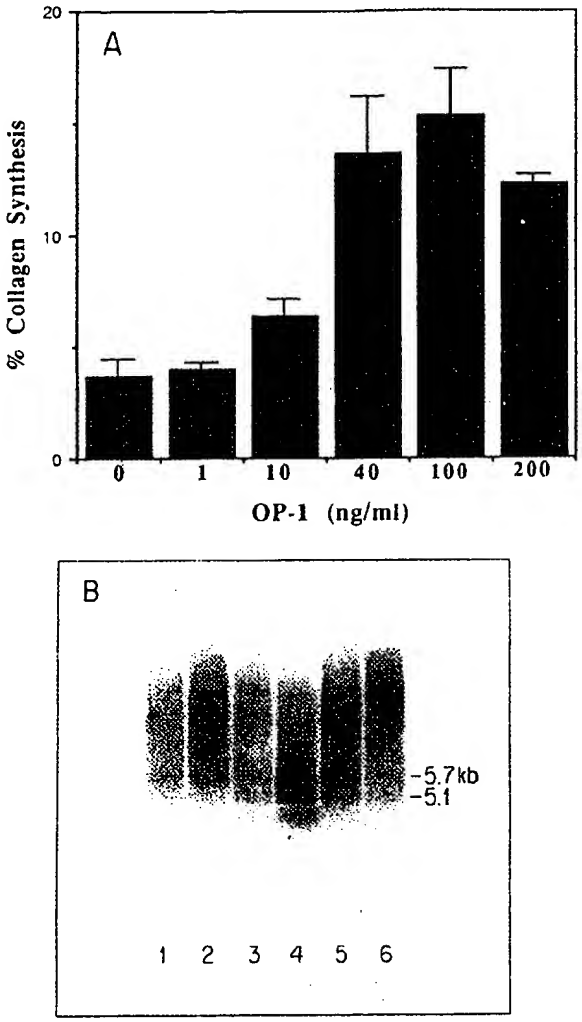

FIGURE 2. (A) Effect of OP-1 on collagen synthesis in ROS 17/2.8 cells. Cultures were grown to confluence, at which time the medium was replaced with serum free medium containing OP-1. After 72 h [^{3}H] proline was added to cultures for the final 6 h, and protein synthesis was measured. The values are means ±SD of triplicate cultures. (B) Northern blot analysis of ROS cell cultures treated with OP-1 for the expression of Type I collagen mRNA. Cultures were treated with OP-1 for 7 days in the presence of 5% serum. 20 μg of total RNA from each culture was loaded per lane for electrophoresis in 1% agarose/formaldehyde gel and blotted. Hybridizations were carried out in a roller bottle apparatus at 1 rev/min for 16 h at 45°C. Lanes: (1) control culture with no treatment; (2) culture treated with OP-1 1 ng/ml; (3) 10 ng/ml; (4) 40 ng/ml; (5) 100 ng/ml; (6) 200 ng/ml.

DISCUSSION

We have previously shown that recombinant OP-1 promotes cell growth and stimulates the expression of markers that are characteristics of the osteoblast phenotype in primary osteoblast-enriched cultures derived from newborn rat calvaria (Sampath et al., 1992). Since it is generally regarded that the primary cultures prepared through sequential collagenase digestion of newborn rat calvaria contain a mixture of chondro/osteoprogenitor cells and osteoblasts at various stage of development (Asahina et al., 1993), we examined the direct effects of hOP-1 on clonal osteoblastic cells. The present study demonstrates that purified recombinant human OP-1 suppresses cell growth and stimulates expression of the osteoblast phenotype in clonal rat osteosarcoma ROS 17/2.8 cells.

The present finding that OP-1 inhibits proliferation of ROS 17/2.8 cells is contrary to the earlier observation in which OP-1 was shown to promote growth of primary rat calvarial cells in osteoblast-enriched cultures (Sampath et al., 1992). It is generally accepted that in osteoblast-enriched cultures, precursor cells at different stages of development undergo cell proliferation prior to their commitment to the mature osteoblast phenotype, and that proliferation slows or ceases during expression of the osteoblast phenotype (e.g. Owen et al., 1990). It is likely that OP-1 promotes growth of uncommitted chondro/osteoprogenitors as seen in rat calvarial cultures (Sampath et al., 1992; Asahina et al., 1993),

TABLE 1
Effect of OP-1 on cAMP production in response to PTH in rat osteosarcoma ROS 17/2.8 culture

	Amounts of cAMP produced (pmol/well)		
OP-1 (ng/ml)	PTH(−)	PTH(+)	PTH(+)/PTH(−) ratio
0	1.71 ± 0.12	6.35 ± 0.21	3.71
1	1.68 ± 0.10	7.20 ± 1.60	4.20
10	1.83 ± 0.28	11.00 ± 1.40	6.00
40	1.88 ± 0.12	33.50 ± 2.10	17.80
100	1.86 ± 0.30	35.33 ± 2.30	18.82
200	1.62 ± 0.16	66.00 ± 5.60	40.74
1000	1.65 ± 0.13	84.50 ± 0.71	51.21

Mean ± SD.

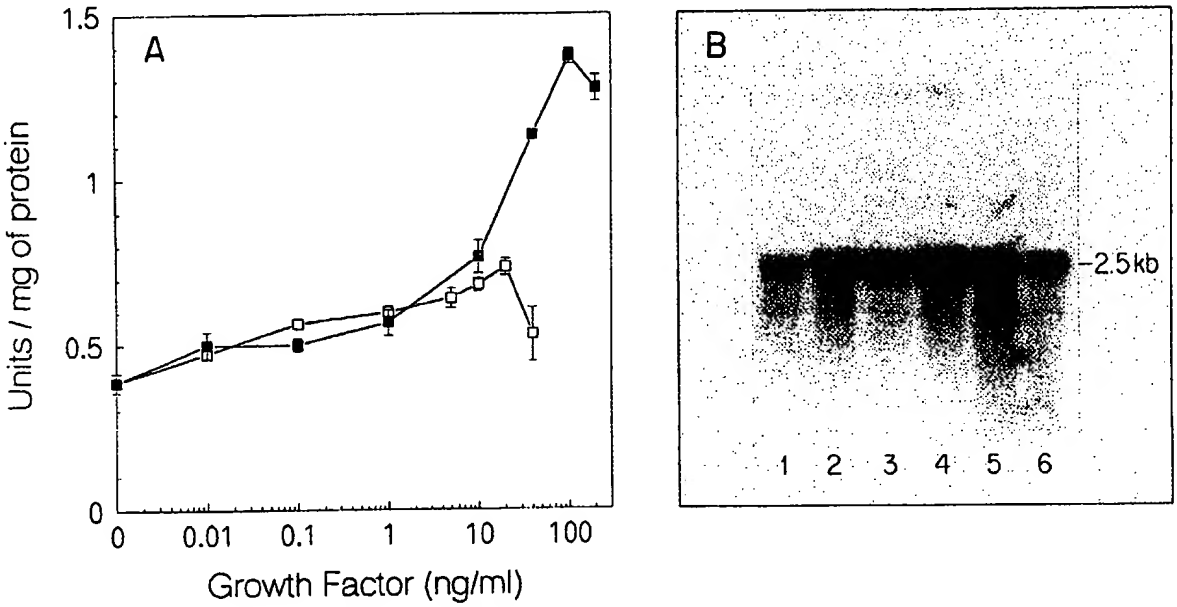

FIGURE 3. (A) Effect of OP-1 and TGF-β1 on stimulation of alkaline phosphatase activity in ROS 17/2.8 cells. Confluent cultures (24-well plates) containing 1% FBS were treated with OP-1 (■) or TGF β1 (□) for 72 h. The cells were extracted and assayed for alkaline phosphatase specific activity (methods). Values are means ± S.D. of triplicate cultures. (B) Northern blot analysis of alkaline phosphatase mRNA in ROS 17/2.8 cell cultures treated with OP-1. The experimental procedures are same as described in Fig. 2B. Lanes: (1) Control culture; (2) culture treated with 1 ng/ml OP-1; (3) 10 ng/ml; (4) 40 ng/ml; (5) 100 ng/ml; (6) 200 ng/ml.

and inhibits cell growth of committed osteoblastic ROS cells. This is consistent with the general rule of an inverse relationship between cell proliferation and differentiation. We have observed similar OP-1 effects in MC3T3-E1, a clonal osteoblastic cell line (unpublished observations). TGF-β1 also appears to act similarly both in rat calvarial cultures and in ROS 17/2.8 cultures.

OP-1 inhibits DNA synthesis in a dose-dependent manner in both the absence and in the presence of serum. The maximal inhibitory effect of OP-1 in serum-free medium occurs at 20 ng/ml in serum-free conditions and at 100 ng/ml in the presence of serum. It is plausible that OP-1 may bind to serum proteins and therefore be less readily available to the cells. TGF-β1 inhibits the growth of ROS 17/2.8 cells to a lesser degree than OP-1 in the absence of serum, while it does not significantly affect growth in the presence of serum, possibly due to endogenous TGF β. The growth-arresting effects of OP-1 on mature osteoblasts are consistent with reports using related bone morphogenetic proteins BMP-2, BMP-3 (osteogenin) and BMP-4 (Vukicevic et al., 1989; Chen et al., 1991; Takuwa et al., 1991).

OP-1 enhances the synthesis of collagen as measured by collagenase digestible protein in a dose-dependent manner and the maximum synthesis occurs at 100 ng/ml. This OP-1 induction of collagen synthesis is further confirmed by the increase in type I collagen mRNA. OP-1 effects on collagen synthesis appear to be specific to the osteoblast. We have observed that OP-1 does not stimulate collagen synthesis in fibroblast cultures under conditions where TGF-β1 is known to enhance matrix synthesis (unpublished data). Recombinant BMP-2, on the other hand, is reported to have no effect on the expression of type I collagen mRNA or collagen synthesis by osteoblast-like cells, calvarial cells and embryonic fibroblasts in culture (Yamaguchi et al., 1991). However, in contrast to BMP-2, a naturally purified BMP-3 preparation (osteogenin) and recombinant BMP-4 were shown to enhance type I collagen synthesis in osteoblast-enriched cultures (Vukicevic et al., 1989; Chen et al., 1991).

ROS 17/2.8 cells are known to express most osteoblast markers including alkaline phosphatase, intracellular cAMP production in response to PTH and osteocalcin synthesis. In support of our earlier observation that OP-1 upregulates expression of the osteoblast phenotype in rat osteoblast-enriched cultures, the present studies with ROS 17/2.8 cells show that OP-1 clearly enhances induction of

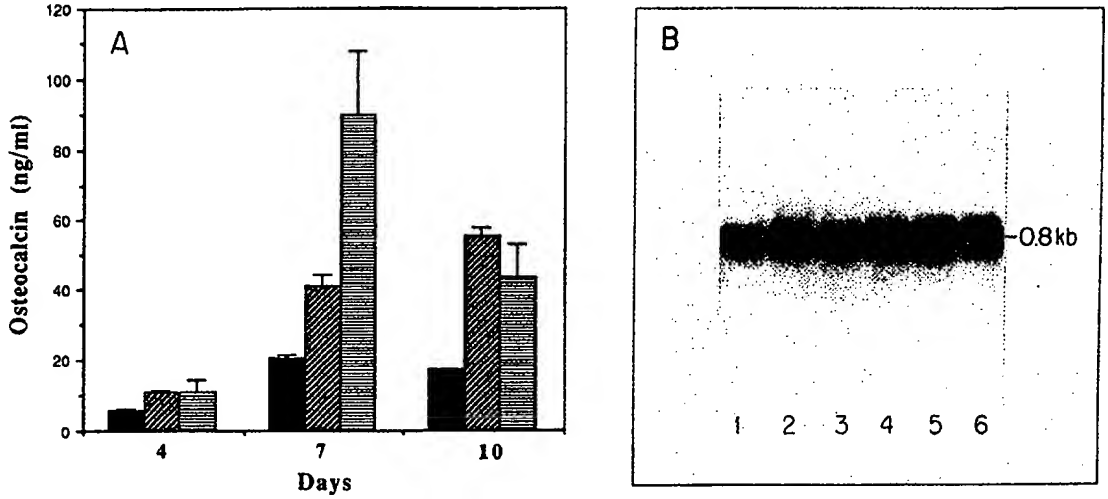

FIGURE 4. (A) Effect of OP-1 on osteocalcin synthesis in ROS cell cultures. Confluent cultures (24-well plates) cultivated in medium containing 5% FBS were treated with 40 ng/ml of OP-1 alone (▨) and in combination with 50 μg/ml of ascorbic acid, and 10 mM β-glycerophosphate (▤) for 4, 7 and 10 days. Control cultures (■) received equal volumes of the OP-1 solvent vehicle. Osteocalcin in the medium was measured by radioimmunoassay and represented as ng/ml culture medium. Values are means ± S.D. of triplicate cultures. (B) Northern blot analysis of osteocalcin mRNA expression in ROS 17/2.8 cell cultures treated with OP-1. Experimental procedures are as described in Fig. 2B. Lanes: (1) control culture with vehicle; (2) culture treated with 1 ng/ml OP-1; (3) 10 ng/ml OP-1; (4) 40 ng/ml of OP-1; (5) 100 ng/ml OP-1; (6) 200 ng/ml OP-1.

alkaline phosphatase specific activity, adenylate cyclase activity in response to PTH, and osteocalcin synthesis. The finding that combinations of OP-1 and TGF-β1 showed an additive effect on the stimulation of alkaline phosphatase activity suggests that these two growth factors may be acting through their respective receptors. We recently observed that OP-1 and TGF-β1 have distinct membrane binding sites in ROS 17/2.8 cells which are not in competition for the two factors (unpublished observation). Furthermore, we have recently shown that OP-1 does not interact with type I, II or III TGF-β specific receptors but with a related receptor that has been shown to be specific to OP-1 (ten Dijke et al., 1994). In summary, we have presented evidence that OP-1, a bone morphogenetic protein member of the TGF-β superfamily, is capable of stimulating matrix synthesis and enhancing the expression of markers that are characteristic of the osteoblast phenotype in a clonal osteoblastic ROS 17/2.8 cells.

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